

**ENVIRONMENTAL INFLUENCES ON VIRULENCE FACTORS
IN *VIBRIO VULNIFICUS***

A Dissertation

by

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ABSTRACT

Understanding the environmental factors influencing virulence of *V. vulnificus* is of utmost importance in reducing human exposure to this deadly pathogen. Quantitative PCR assays for the virulence indicator genes *vcg* and 16S rRNA type A/B were used to examine fluctuations in virulence with season in Galveston Bay oysters. A strong seasonal shift of *V. vulnificus* strain types was observed. Environmental strains (16S rRNA type A) predominated from April to mid-June as temperatures rose 20 to 28°C and salinities increased from 22 to 27 PSU. As temperatures increased to $\geq 30^{\circ}\text{C}$ from mid-June to September and salinities rose above 27 PSU, clinical strains (16S rRNA type B; *vcgC*) predominated.

Enrichment media was tested for selective enrichment for environmental or clinical strains from seawater. Ratios of clinical to environmental strains enriched in alkaline peptone water (APW) remained constant, however Brain Heart Infusion Broth (BHIB), with its higher iron content, preferentially enriched for clinical strains indicating that iron plays an important role in the infection of the human host.

Biofilm formation by clinical and environmental reference strains of *V. vulnificus* were tested under different temperature pH and iron concentration conditions. Clinical strains were found to produce significantly more biofilm than environmental strains at 24°C versus 30°C or 37°C. There was a significant trend for clinical strains of *V. vulnificus* to form more biofilm at acidic pH (5.5) than environmental strains, at both 24°C and 37°C, indicating that biofilm may be important for survival of clinical strains in the gastrointestinal tract of the human host. There was a strong, direct correlation

between iron concentration in the growth medium and biofilm production by all strains tested. With regards to temperature and pH, higher biofilm production appears to be a trait of clinical strains and could be considered a virulence factor.

Examination of genes encoding for virulence factors hemolysin/cytolysin and capsular polysaccharide during biofilm formation revealed that their expression was influenced by maturity of the biofilm as well as by temperature. Both genes were up-regulated during biofilm formation indicating that biofilm may be the preferred form of *V. vulnificus* in the human host.

DEDICATION

To my mom

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NOMENCLATURE

CPS	Capsular polysaccharide
DNA	Deoxyribonucleic acid
h	Hour
LPS	Lipopolysaccharide
OD	Optical density
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
PSU	Practical salinity units
RNA	Ribonucleic acid
rRNA	Ribosomal RNA

TABLE OF CONTENTS

	Page
ABSTRACT	ii
DEDICATION	iv
ACKNOWLEDGEMENTS	v
NOMENCLATURE.....	vi
TABLE OF CONTENTS	vii
LIST OF FIGURES.....	ix
LIST OF TABLES	xi
CHAPTER I INTRODUCTION	1
CHAPTER II LITERATURE REVIEW	6
<i>Vibrio vulnificus</i> ecology	6
Early clinical investigations	7
<i>Vibrio vulnificus</i> disease.....	8
Isolation and detection methods	10
Classification systems for strains	11
Virulence factors and pathogenicity	14
Biofilm and <i>V. vulnificus</i>	25
CHAPTER III QUANTITATIVE PCR ENUMERATION OF VCGC AND 16S RRNA TYPE A/B GENES AS VIRULENCE INDICATORS FOR ENVIRONMENTAL AND CLINICAL STRAINS OF VIBRIO VULNIFICUS IN GALVESTON BAY OYSTERS	33
Overview	33
Introduction	34
Materials and methods	37
Results	41
Discussion	44
CHAPTER IV SELECTIVE ENRICHMENT FOR CLINICAL STRAINS OF	

VIBRIO VULNIFICUS	51
Overview	51
Introduction	51
Materials and methods	55
Results	58
Discussion	63
CHAPTER V THE EFFECTS OF TEMPERATURE, PH, AND IRON ON BIOFILM FORMATION BY VIBRIO VULNIFICUS.....	67
Overview	67
Introduction	68
Materials and methods	72
Results	76
Discussion	94
CHAPTER VI DIFFERENTIAL EXPRESSION OF VVHA AND CPS OPERON ALLELE 1 GENES IN VIBRIO VULNIFICUS UNDER BIOFILM AND PLANKTONIC CONDITIONS	100
Overview	100
Introduction	101
Materials and methods	104
Results	108
Discussion	114
CHAPTER VII SUMMARY AND CONCLUSIONS.....	119
Seasonal variation of virulence	119
Selection of enrichment media for more virulent strains	121
Biofilm formation	123
Expression of virulence factors in biofilms.....	124
REFERENCES	125

LIST OF FIGURES

	Page
Figure 1 The five stages of biofilm development.....	27
Figure 2 Temperature (°C) and salinity (PSU) in seawater at Sammy's Reef between 12/8/09 and 12/8/10	42
Figure 3 CFU/g a) <i>vvhA</i> , b) 16S rRNA types A and B, and c) <i>vcgC</i> in Sammy's Reef oysters between 12/8/09 and 12/8/10	45
Figure 4 Proportion (%) of genes a) 16S rRNA type A, b) 16S rRNA type B, and c) <i>vcgC</i> out of the total <i>V. vulnificus</i> pool as determined by quantification of <i>vvhA</i> genes	46
Figure 5 Correlation of 16S rRNA type B with <i>vcgC</i> CFU/g	47
Figure 6 Concentration of <i>vvhA</i> (CFU/ml) and % <i>vcgC</i> genes in enrichment media APW10 and BHIB10	59
Figure 7 <i>VvhA</i> CFU/ml in seawater enriched with APW10, APW5, BHIB10, BHIB5, and LB10 for samples collected 3/22/12, 9/4/12, and 11/4/12 ...	61
Figure 8 Percent <i>vcgC</i> in seawater enriched with APW10, APW5, BHIB10, BHIB5, and LB10 for samples collected 3/22/12, 9/4/12, and 11/4/12 ...	62
Figure 9 Biofilm stained with 0.1% crystal violet	75
Figure 10 Effect of temperature on biofilm production by environmental (numbers) and clinical (letters) strains (trial 1).....	78
Figure 11 Effect of temperature (°C) on biofilm production by environmental (numbers) and clinical (letters) strains (trial 2).....	79
Figure 12 Effect of temperature (°C) on biofilm production by environmental (numbers) and clinical (letters) strains (trial 3).....	80
Figure 13 Biofilm production by environmental (numbers) and clinical (letters) strains of <i>V. vulnificus</i> at different pH (trial 1).....	83

Figure 14 Biofilm production by environmental (numbers) and clinical (letters) strains of <i>V. vulnificus</i> at different pH (trial 2)	84
Figure 15 Biofilm production by environmental (numbers) and clinical (letters) strains of <i>V. vulnificus</i> at different pH (trial 3)	85
Figure 16 Biofilm production by environmental (numbers) and clinical (letters) strains of <i>V. vulnificus</i> at different pH incubated at 37°C (trial 1).....	88
Figure 17 Biofilm production by environmental (numbers) and clinical (letters) strains of <i>V. vulnificus</i> at different pH incubated at 37°C (trial 2).....	89
Figure 18 Biofilm production by environmental (numbers) and clinical (letters) strains of <i>V. vulnificus</i> with increasing ferric chloride concentrations according to regression analysis (trial 1).....	91
Figure 19 Biofilm production by environmental (numbers) and clinical (letters) strains of <i>V. vulnificus</i> with increasing ferric chloride concentrations (trial 2)	92
Figure 20 <i>V. vulnificus</i> isolate FLA 8869 incubated at 24°C for 24 h.....	112
Figure 21 <i>V. vulnificus</i> isolate FLA 8869 incubated at 37°C for 24 h.....	113

LIST OF TABLES

	Page
Table 1 Genotyping PCR assays for virulence factors and type strains.....	16
Table 2 Gene clusters involved in synthesis of CPS in <i>V. vulnificus</i>	20
Table 3 Primers and probes used to quantify virulence factor genes in <i>Vibrio vulnificus</i>	41
Table 4 <i>V. vulnificus</i> strains used in this study.....	72
Table 5 Effect of temperature on biofilm production by environmental (numbers) and clinical strains (letters) (trial 1)	78
Table 6 Effect of temperature on biofilm production by environmental (numbers) and clinical strains (letters) (trial 2)	79
Table 7 Effect of temperature on biofilm production by environmental (numbers) and clinical strains (letters) (trial 3)	80
Table 8 Comparison of biofilm production at different temperatures (trial 1)	81
Table 9 Comparison of biofilm production at different temperatures (trial 2)	81
Table 10 Comparison of biofilm production at different temperatures (trial 3)	81
Table 11 Effect of pH on biofilm production by three environmental (numbers) and three clinical (letters) strains (trial 1)	83
Table 12 Effect of pH on biofilm production by three environmental (numbers) and three clinical (letters) strains (trial 2)	84
Table 13 Effect of pH on biofilm production by three environmental (numbers) and three clinical (letters) strains (trial 3)	85
Table 14 Biofilm production by clinical vs environmental strains at different pH (trial 1)	86
Table 15 Biofilm production by clinical vs environmental strains at different pH (trial 2)	86

Table 16 Biofilm production by clinical vs environmental strains at different pH (trial 3)	86
Table 17 Effect of pH incubated at 37°C on biofilm production by three environmental (numbers) and three clinical (letters) (trial 1)	88
Table 18 Effect of pH incubated at 37°C on biofilm production by three environmental (numbers) and three clinical (letters) (trial 2)	89
Table 19 Biofilm production by clinical vs environmental strains at different pH incubated at 37°C (trial 1).....	90
Table 20 Biofilm production by clinical vs environmental strains at different pH incubated at 37°C (trial 2).....	90
Table 21 Effect of ferric chloride on biofilm production by environmental (numbers) and clinical (letters) strains (trial 1)	91
Table 22 Effect of ferric chloride on biofilm production by environmental (numbers) and clinical (letters) strains (trial 2)	92
Table 23 Growth rates (generations/hr) of environmental (numbers) and clinical (letters) strains grown at different temperatures	93
Table 24 Growth rates (generations/hr) of environmental (numbers) and clinical (letters) strains grown at different pH	93
Table 25 Up (+) or down (-) regulation of <i>vvhA</i> and <i>CPS allele 1</i> genes normalized to individual cells during biofilm formation	114

CHAPTER I

INTRODUCTION

Vibrio vulnificus are Gram-negative, motile, halophilic curved rod-shaped bacteria which naturally reside in coastal marine environments worldwide. It is considered as one of the most invasive and fatal pathogenic bacteria to humans and has been isolated from water, sediments, shellfish such as oyster, mussels, shrimp, fish, and clams (1-7). Even if the number of *V. vulnificus* infected individuals is lower than that of other vibrios, it accounts for 95% of all seafood related deaths in the United States, and causes the highest death rate of any foodborne disease (8-11). It is the causative agent of two types of diseases, fulminant primary septicemia and necrotizing wound infections. The type of disease and the number of deaths depend on the route of transmission. Consumption of raw or undercooked seafood containing a high enough concentration of *V. vulnificus* can cause primary septicemia with a death rate exceeding 50% in people having an underlying disease, in particular diseases that elevate serum blood levels such as hemochromatosis, liver cirrhosis, and diabetes (11, 12). It can also cause severe wound infections by exposure of pre-existing wounds to seawater harboring this bacterium, with a mortality rate up to 25% (2, 13). The majority of cases results in death within 24-48 hours. Interestingly, males are more susceptible to this bacterium than female and most (95%) of cases have been observed in males, because estrogen provides protection against *V. vulnificus* endotoxin in females (14).

General ecology and distribution depend on several factors, including temperature, salinity, dissolved organic matter, oxygen, and pH, but it has been reported that temperature and salinity are the most important determinant affecting the occurrence of *V. vulnificus* (15-17). *V. vulnificus* is halophilic and is most prevalent in seawater having salinities between 5 and 25 PSU (18, 19). And, it grows best at temperatures between 10°C and 30°C (16, 19). This bacterium encounters several environmental stresses in its natural habitat, such as high or low temperature, limited nutrient availability, oxidative stress, and fluctuations in salinity. To cope with these challenges, *V. vulnificus* enters a viable-but-nonculturable state (VBNC), especially during cold temperatures below 10°C (20). *V. vulnificus* in the VBNC state are not able to grow, even on nutrient rich medium, so the incidence of infection is almost zero during the winter (21). Most cases of infection happen between May and October when seawater temperatures typically range between 20°C and 30°C (22).

V. vulnificus exhibits varied degrees of virulence. Strains isolated from hospital patients, referred to as ‘clinical,’ are highly virulent, whereas strains isolated from seawater, oysters, and other marine life are typically less virulent (23, 24). One of the first studies (23) to observe differences in virulence of clinical versus environmental strains using iron-overloaded mice found that environmentally isolated strains required a 350-fold higher inoculum than clinical strains for infection. And, death occurs more frequently in patients infected with clinical strains rather than environmental strains (25-27).

Although the pathogenic mechanism of this bacterium has not been thoroughly elucidated, various virulence factors have been identified such as capsular polysaccharide (CPS) (28), lipopolysaccharide (LPS) (29), iron uptake system (30), pili (31), flagella (32), outer membrane proteins (33), rtxA toxin (34), hemolysin/cytolysin (35) and metalloprotease (36). Even though all of these virulence factors facilitate the virulence of *V. vulnificus*, CPS is considered the most important virulence determinant because it provides protection from the non-specific host immune system (37).

Molecular typing methods for several virulence markers have been developed to distinguish between clinical and environmental strains (38-42). Some of these virulence markers such as LPS (29), CPS (41), flagella (32), hemolysin/cytolysin (43) and metalloprotease (44) cannot completely distinguish the clinical from environmental strains. However others, such as 16S rRNA (type A and B) (40), the *viuB* gene that encodes for vulnibactin (siderophore) (41) and the virulence correlated gene (*vcg*) (39), are have shown promise.

Chapter II of this dissertation is an in depth literature review of *V. vulnificus* ecology disease, virulence, and biofilm formation.

Chapter III evaluates the application of two virulence factor genotypes, 16S rRNA (type A and B) and the clinical variant of the *vcg* gene, to study the seasonal variation of clinical and environmental strains of *V. vulnificus* in oysters. 16S rRNA type B is associated with clinical strains and type A with environmental strains. Good agreement was observed by Nilsson et al. (25) who examined clinical isolates from patient fatalities (94% type B), patient recoveries (50% type B), and environmental

strains from oysters or seawater (94% type A). *Vcg* has two sequence variants, *vcgC* and *vcgE*. The *vcgC* variant has a 90% accurate detection rate for known clinical isolates whereas the *vcgE* variant has an 87% detection rate for known environmental isolates (39). The following hypotheses were tested:

H_0 : There is no seasonal variation in populations of clinical and environmental strains of *V. vulnificus*;

H_1 : The *vcgC* gene, a highly accurate indicator of clinical strains in isolate cultures, can accurately detect clinical strains in environmental samples.

Chapter IV tests liquid media typically used to maintain *V. vulnificus* isolate cultures or to enrich for *V. vulnificus* prior to plating on selective-differential agars for selective enrichment of clinical versus environmental strains. Standard isolation methods for *V. vulnificus* involve a prior enrichment step with Alkaline Peptone Water (APW) followed by streaking onto selective-differential agars. The question is whether APW selects preferentially for clinical or environmental strains. The following hypotheses were tested:

H_1 : APW does not select equally for clinical and environmental strains thereby altering the isolation rate of each on selective-differential agars.

H_2 : Brain Heart Infusion Broth with its high iron content selects preferentially for clinical strains similar to the human host.

Chapter V examines environmental factors that affect formation of biofilms by *V. vulnificus*. Due to the nature of infections (i.e. blood or wounds), biofilm production by *V. vulnificus* has been largely ignored except for a few studies that identified genes

responsible for biofilm formation (31, 45-48). Most studies of biofilm formation in vibrios have been conducted with *V. cholerae*. *V. cholerae* forms robust biofilms in the aquatic environment (49), and in response to bile acids in the gastrointestinal tract (50). The following hypotheses were tested:

H₁: Similar to *V. cholerae*, *V. vulnificus* will produce more biofilm at acidic pH than at neutral or alkaline pH.

H₂: *V. vulnificus* will produce more biofilm at 37°C, the temperature of its human host.

Chapter VI examines the expression of hemolysin/cytolysin (*vvhA*) and CPS operon (*CPS allele 1*) genes during biofilm formation by *V. vulnificus* to determine if either are affected by biofilm production. It has been demonstrated that *V. cholerae* is more virulent in biofilm than in its planktonic form (49, 50). While, CPS, the most important virulence factor, provides protection from the non-specific host immune system (37) it may also be an important component of biofilm in *V. vulnificus* (51). However, Joseph and Wright (45) found that CPS expression inhibits biofilm formation in *V. vulnificus*. The following hypotheses were tested:

H₁: *V. vulnificus* expression of the hemolysin/cytolysin gene (*vvhA*) will be higher during biofilm formation than in planktonic cells.

H₂: Expression of *CPS allele 1* will be suppressed during biofilm formation and increased in planktonic forms of *V. vulnificus*.

Chapter VII is a summary of the four studies.

CHAPTER II

LITERATURE REVIEW

Vibrio vulnificus ecology

V. vulnificus occurs naturally in estuarine and coastal waters worldwide. It can be isolated from shellfish, especially oyster, mussels, clams, fish, shrimp and sediments (1, 2, 6, 7, 22). Temperature and salinity are the most important factors affecting the distribution of *V. vulnificus*. Concentration of *V. vulnificus* in seawater and oysters fluctuate with seasonal temperature changes. Typically, during the winter when seawater temperatures fall below 10°C *V. vulnificus* is almost undetectable (5, 52, 53) and it enters a state of viable-but-nonculturable (20). As seawater temperature increases above 10°C in spring months and approaches 25°C in the early summer the concentration of *V. vulnificus* increases to $\sim 10^1$ - 10^2 CFU/ml and can reach 10^3 CFU/ml when seawater temperatures rise above 25°C during summer months (16, 19, 53). *V. vulnificus* is not able to multiply in oysters at 13°C or lower temperatures and concentrations are typically ≤ 10 CFU/g oyster meat (15, 54) but during warmer months, as temperatures reach 25°C and above, concentrations increase to $>10^3$ CFU/g (15).

Highest *V. vulnificus* concentrations (10^3 CFU/ml) can be detected in seawater with salinity from 5 to 30 PSU (19). The highest number in oysters ($>10^3$ CFU/g) typically occurs at salinities between 5 and 25 PSU but concentrations decrease at higher salinities (15).

Early clinical investigations

Hollis et al. (55) with the Special Bacteriology Section of the Centers for Disease Control (CDC) in Atlanta, Georgia, published the first clinical description of *V. vulnificus* from extensive diagnostic biochemical testing of 38 strains of “unnamed marine *Vibrio*.” Most were obtained over a period of 11 years from medical case studies in 15 US states (seven in LA, six in FL, five in AL, four in TX, four in RI, two in HI, two in VA, one in NC, one in AR, one in GA, one in CA, one in SC, one in WI, one in MA, and one in PA). The strains were isolated from blood (20 cases), spinal fluid (2 cases), and localized limb infections (16 cases). Patients recently had either ingested raw/undercooked seafood (56) or been exposed to seawater (57-59). Prior to testing at the CDC, it was widely speculated that this new vibrio was a “biotype” of *V. parahaemolyticus*, (60) or *V. alginolyticus*, referred to then as “*V. parahaemolyticus* biotype 2 (*alginolyticus*)” (61). However, unlike either of these vibrios, this new strain was capable of lactose fermentation and thereafter was referred to as “lactose positive” (55) until it was taxonomically described and formally give the name “*Vibrio vulnificus*” (62, 63).

Galveston Bay (Texas) figured prominently in these early investigations of *V. vulnificus*. Of the 38 ‘clinical’ strains tested by the CDC (55), four from Texas were isolated from patients who had become ill after exposure to seawater in Galveston Bay and the nearby Gulf of Mexico (58). Just a few years later, after several cases of bacteremia (two fatal) on Galveston Island, a 12 month study was initiated to determine the distribution and occurrence of *V. vulnificus* at 21 sites typically frequented by

tourists and local inhabitants (64). Sites located on the bay side of the island, including the ferry landing, ship harbor, bayou, and yacht basin, were 67 to 100% positive for *V. vulnificus* throughout the study period whereas beach sites on the open gulf side of the island had significantly lower occurrences (0 to 17%). The more enclosed yacht basin and ship harbor had the highest occurrence rates of 71 and 100%, respectively. Seven ‘environmental’ strains originating from this study were later used by Johnson and Calia (65) in the first tests for hemolytic activity. No bacteriological differences were observed between ‘clinical’ (three fatal bacteremia, one non-fatal bacteremia) plus another isolated from an oyster, and the ‘environmental’ strains. However the clinical strains demonstrated significantly greater serum resistance (a zero decline of \log_{10} CFU after 2 hr vs. 2.6 \log_{10} decline of CFU in environmental strains) as well as virulence (1,000 times lower LD_{50} than environmental strains) in orogastric challenge tests with suckling mice (66).

In the 25 years that have followed, characterization of *V. vulnificus* has taken divergent paths: 1) medical diagnosis 2) molecular identification of virulence factors for development of antibiotics and vaccines, 3) seafood safety for the prevention of illness, and 4) ecological to understand the environmental conditions for growth and virulence.

***Vibrio vulnificus* disease**

V. vulnificus accounts for 95% of all seafood related deaths in the United States, and causing the highest-death rate of any food-borne disease (8, 21). Forms of illness after ingestion of *V. vulnificus* are typically gastrointestinal (gastroenteritis) presented as

vomiting, diarrhea, and severe abdominal pain, but if not treated, can rapidly infect the bloodstream (septicemia) within 24 to 48 hr resulting in fever, chills, blistering skin lesions (bullae), tissue necrosis, and death (2, 13, 67). Infections can also be caused by exposure of pre-existing or fresh wounds to seawater containing *V. vulnificus* or by handling seafood (2). Both the type of disease and the number of deaths depend on the route of transmission. The mortality rate for primary septicemia, the most lethal form of infection, is greater than 50% after consumption of raw or undercooked oysters having high enough titers of *V. vulnificus* cells, and it can result in higher mortality rate in people who are immuno-compromised or have an underlying disease such as cancer, diabetes, hepatic disorders, hemochromatosis, thalassemia, and alcoholism (12). The leading cause of septicemia in those immunocompromised people is due to the elevated iron levels in blood. People with compromised immune systems or chronic liver disease are also at risk and 80 times more likely than healthy people become septicemic (68). However, primary septicemia is quite rare in healthy individuals. After first indications of infection, death from septicemia typically occurs within one or two days (37, 68). Exposure of pre-existing or fresh wounds to seawater containing *V. vulnificus* can also lead to infection and a 25% mortality rate in people with wound infections has been observed (13). Wound infection is thought to occur as a consequence of seafood handling, fishing, boating, swimming, or wading (2, 67). Most cases of wound and ingestion infection happen between May and October when the temperature of seawater is warmer (20°C to 30°C) (22).

Isolation and detection methods

In the clinical setting *V. vulnificus* from tissues and blood are isolated on blood agars (69). Standard methods for enumeration and isolation of *V. vulnificus* from environmental samples involve enrichment in Alkaline Peptone Water (APW) followed by streaking onto selective differential agars. Several media agars have been developed such as thiosulphate citrate bile salts sucrose TCSB (70) sodium dodecyl sulfate-polymyxin B-sucrose agar (SPS) (71), *V. vulnificus* (VV) agar (72), *V. vulnificus* enumeration medium (VVE) (73), *V. vulnificus* medium (VVM) (74), cellobiose polymyxin B colistin (CPC) agar (73), modified mCPC (75). Cultivation based-detection and enumeration methods do not meet the necessary sensitivity and specificity reliability, therefore identification of the strains is followed by biochemical, immunological assays (75), DNA probes (76) and most recently-developed molecular polymerase chain reaction (PCR) assays (77).

A quantitative real-time PCR based on TaqMan oligonucleotide fluorogenic probe targeted on *V. vulnificus* hemolysin/cytolysin-encoding gene, *vvhA*, has been developed for direct detection and enumeration of *V. vulnificus* with a detection threshold of 6 CFU per PCR assay or 72 fg per microliter of PCR mixture from pure cultures (78). Probe-based quantitative real-time PCR makes use of a specific hybridization probe having a reporter fluorescent dye at 5' end and a quencher molecule at 3' end. When the probe is in its native state no fluorescence is emitted, because the quencher molecule absorbs the emission of reporter dye due to the close proximity of both molecules, but during amplification the 5'-3' exonuclease activity of *Taq*

polymerases releases the reporter from the vicinity of quencher and causes an increase in fluorescence (79). SYBR Green I-based real-time quantitative PCR assay was described based upon *vvh*-specific oligonucleotide primers with the detection rate equivalent to 1 pg of purified DNA or 10^2 cells in 1 g of unenriched oyster homogenate or 10 ml of seawater samples (80). SYBR Green I based RT-PCR assay utilizes fluorogenic, DNA intercalating SYBR green dye which binds the minor groove of double stranded DNA during amplification of the products of interest, and the biggest advantage of this assay is the use of any primer set which can, if optimally designed, can preclude non-specific targets in the reaction mixtures (79, 81). The drawback of SYBR green dye is the binding of any dsDNA of target and non-target products, which can be overcome by melting curve analysis the melting curve peaks of which will differ in target and non-target amplicons (79).

Classification systems for strains

Classification of *V. vulnificus* is currently based on 1) biotype, 2) genotyping, and 3) virulence factors such as siderophore production, lipopolysaccharides (LPS), capsule polysaccharides (CPS), and hemolytic/cytolytic enzymes.

Biotypes. *V. vulnificus* strains have been more formally divided into three distinct biotypes based upon their biochemical and phenotypic characteristics (68).

Biotype 1 strains are almost completely related to human disease and generally found in association with seawater and shellfish. These strains are positive for indole and

ornithine decarboxylase production and produce many immunologically different types of LPS. *Biotype 2* strains mainly cause disease in marine vertebrates, especially in eels, and are not able to produce indole and ornithine decarboxylase and produce only a single type of lipopolysaccharide (LPS), designated as “*serogroup E*,” A third biotype, *Biotype 3* was identified in Israel as strains isolated from wound infections and septicemic individuals that handle cultured *tilapia* (2). Comparison of these three biotypes based on genetic analysis has revealed that *Biotype 3* is a hybrid of *Biotypes 1* and *2* and phenotypically differs from them by the inability to ferment salicin, cellobiose, and lactose (68).

Genotyping. Several genes that differentiate clinical versus environmental strains have been identified—16S rRNA (type A/B), virulence correlating gene (*vcg*), siderophore gene (*viuB*), pilus-type IV gene (*pilF*), and capsulated polysaccharide (*CPS alleles 1 and 2*).

The 16S small subunit (SSU) ribosomal ribonucleic acids (rRNA), 23S large subunit (LSU) rRNA, and the interspacer (ITS) region between these two genes allows for differentiation between clinical and environmental strains. Analysis of 16S rRNA determined that the “*B type*” was indicative of clinical strains whereas environmental strains were “*A type*” (38, 82). Using terminal restriction length polymorphism (TRFLP) and quantitative PCR, Vickery et al. (27) was able to positively identify 97% of known environmental isolates (from oysters and water) as 16s rRNA type A, however 29% of these were later determined to be both type A and B. And, they determined that 76% of

known clinical strains were type B, with 14% of these being both type A and B. Interestingly, the majority of the type A/B clinical strains were isolated from patients who recovered from *V. vulnificus* infection whereas the type B strains were isolated from patient fatalities. Typing analysis of conserved sequences located between the 16S and 23S rRNA genes (interspacer region) revealed that clinical and environmental strains can be grouped into a single clusters, the first consisting of highly similar clinical strains of type B but the cluster of environmental strains is a combination of type A and B (83).

The virulence correlating gene (*vcg*), a 200 bp noncoding region identified by Rosche et al. (39) with random amplified polymorphic DNA (RAPD) PCR has two sequence variants—one that occurs primarily in clinical strains (*vcgC*) and another that occurs primarily in environmental strains (*vcgE*). Rosche determined a 90% identification rate for *vcgC* with known clinical strains and a 93% rate for *vcgE* with known environmental strains.

Siderophore production differs between virulent and less virulent *V. vulnificus* strains. Panicker (84) identified a 504 bp gene, *viuB*, that encodes for the siderophore vulnibactin in *V. vulnificus*. The *viuB* gene was found to occur in 100% of known clinical isolates tested and in only 24% of known environmental isolates tested. However, another study (41) analyzed 349 *V. vulnificus* oyster isolates for presence/absence of the *viuB* gene and found that only 41% of known clinical strains tested positive indicating that this gene is not reliable for differentiation.

Roig et al. (42) proposed that the polymorphism in *pilF* gene encoding for pilus-type IV can be used as a virulence marker to distinguish less virulent environmental

strains from more virulent clinical strains of *V. vulnificus*, regardless of the biotypes of strains. Baker-Austin (85) later developed a quantitative PCR assay for the clinical variant of the *pilF* gene and found a 97.9% association with known clinical strains.

The group 1 capsular polysaccharide (CPS) operon has two genotypes, allele 1 that corresponds to clinical strains and allele 2 to environmental strains (86). Allele 1 was found in clinical isolates 63.6% of the time and in environmental isolates 36.4% of the time. Allele 2 was found in environmental isolates 71.4% of the time and in clinical isolates 20% of the time. And, neither allele was present in 8.6% of environmental strains (24). Another study showed that the CPS allele 1 had a 60% detection rate in clinical isolates and allele 2 had a 50% detection rate in environmental isolates (41).

Traditional and quantitative PCR assays have been developed for these genotypes (Table 1).

Virulence factors and pathogenicity

Johnson et al. (66) conducted one of the earliest studies of virulence studies using the original, and not yet named, strains isolated from infected individuals, i.e. “clinical” and seawater, i.e. “environmental.” Individual isolates were inoculated onto blood agar plates to determine susceptibility to bactericidal properties in human blood serum. Most of the clinical strains demonstrated a significantly greater resistance, or survival rate, to human blood serum than environmental strains. No observable die off, or decline, of colony forming units (CFU), expressed as “log₁₀,” occurred in the clinical strains versus an average 2.6 decline of log₁₀ CFUs among environmental strains after a 2 hr exposure

to the sera. The same study observed significantly higher virulence (1,000×) in clinical strains than for the environmental strains during orogastric challenge tests involving inoculating the mouths and stomachs of suckling mice with a dilution series of each isolate. However, in a previous study with the same isolates, Johnson and Calia (65) observed no statistical difference in hemolytic activity between strains from infected individuals or the environment. A more recent study showed that colony opacity was a reliable predictor of virulence in *V. vulnificus* strains, regardless of clinical or environmental source (87). Without exception, opaque colonies, differentiated from translucent colonies when grown on heart infusion agar, were lethal to adult mice at LD₅₀ of 10⁹ (in 0.5 ml intraperitoneal injections) and addition of Fe³⁺ (as ferric ammonium citrate) lowered the LD₅₀ to 10³, indicating that iron availability strongly influences virulence (87). Translucent colonies never caused fatalities, even with addition of iron.

Subsequent studies during the past 25 years have identified several factors influencing the degree of virulence in *V. vulnificus*. It is also becoming increasingly evident that these factors, when present singly or in combination, confer highly variable degrees of virulence

Table 1. Genotyping PCR assays for virulence factors and type strains

Primer/ Probe	Target	Strain Specificity	Primer Sequence (5'-3')	Assay	Ref.
P1 P3	<i>vcg</i>	C/V ¹ , Biotype 1	AGC TGC CGA TAG CGA TCT CGC TTA GGA TGA TCG GTG	qualitative	(39)
P2 P3		E/A ² , Biotype 1	CTC AAT TGA CAA TGA TCT CGCT TAG GAT GAT CGG TG		
Vvu16S51-F Vvu16S221-R Vvu16SA-P vu16SB-P	16S rRNA	mostly Biotype 1 type A type B	CAA GTC GAG CGG CAG CA TCC TGA CGC GAG AGG CC TGA TAG CTT CGG CTC AA CCC GTA GGC ATC ATG C	quantitative	(27)
HP1F HP1R HP2F HP2R	allele 1 for capsule allele 2 for capsule	C/V, Biotype 1 E/A Biotype 1	TTT GGG ATT TGA AAG GCT TG GTG CCT TTG CGA ATT TTG AT TTC CAT CAA ACA TCG CAGA A CTT TTG TCC GGC TTC TAT CG	qualitative	(41)
F-viuB R-viuB	<i>viuB</i> , siderophore	C/V Biotype 1	GGTTGGGCACTAAAGGCAG ATATA CGGCAGTGGACTAATACGCA GC	qualitative	(40)
PilF-F PilF-R PilF-P	<i>pilF</i> , pilus type F	C/V, Biotypes 1, 2, & 3	GAT TGA CTA CGA YCC ACA CCG GRC GCG CTT GGG TGT AG TGC TCA ACC TCG CTA AGT TGG AAA TCG ATA C	quantitative	(85)
vvC-F vvC-R vcgC-P	<i>vcg</i>	C/V Biotype 1	MMA AAC TCA TTG ARC AGT AAC GAA A AGC TGG ATC TAA KCC CAA TGC AAT TAA AGC CGT CAA GCC ACT TGA CTG TAA AGA A	quantitative	(88)

¹ Clinical or virulent strains

² Environmental or avirulent strains

Capsular polysaccharide. Production of an extracellular capsular polysaccharide (CPS) is a key virulence factor for human and animal pathogens. Extracellular acidic polysaccharide capsule (CPS) is deemed as the most significant virulence determinant because CPS allows *V. vulnificus* evade host immune response by avoiding phagocytosis and complement mediated immunity (37, 68). CPS with lipopolysaccharide is thought to play an important role in septic shock formation by stimulating the expression of inflammation-associated cytokines (IL-6 and TNF- α) (89). The differentiation of capsular *V. vulnificus* from the non-capsular one can be done by the observation of colony morphology. While the presence of capsule gives opaque colonies, non-encapsulated strains display translucent colony morphology on media (90, 91). Rosche et al. (92) indicated a third colony that seems to produce an intermediate level of CPS expression, suggesting that intermediate colony possesses opacity between translucent and opaque morphotypes and has a stable phenotype. Decreased expression of CPS highly correlates with the reversible conversion of opaque colonies to translucent colonial morphology. Again opaque colonies are more virulent than translucent strains. The amount of CPS in *V. vulnificus* strains is highly related to the degree of virulence in the animal models (91). Results from this study revealed that the CPS-mutant isolates generated by transposon mutation have an LD₅₀ much higher than wild type strains possessing a polysaccharide capsule (91). CPS mutant strains, created as a consequence of constructed mutation or from naturally occurring phase variation, demonstrated decreased virulence activity in mice model (90, 93-95). Non-encapsulated strains are not protected from human defenses. Shinoda et al. (96) observed the susceptibility of non-

encapsulated mutants to bactericidal effects of human serum. Translucent isolates showed an intermediate level of capsule production; whereas, opaque colonies resulted in an elevated level of expression of CPS during logarithmic growth and the amount of capsule was decreased during stationary phase. It was also observed that a significant amount of CPS was produced at 30°C relative to 37°C (97).

Smith et al. (98) determined the functions of three gene clusters (Table 2) and four unidentifiable ORFs (open reading frames) involved in synthesis of CPS. Studies have shown that mutations in those genes reduce or remove virulence ability of pathogenic bacteria. Four genes in the *wcv* cluster (*wcvA*, *wcvF*, *wcvI*, and *ORF4*) were determined to be essential for synthesis of CPS in *V. vulnificus* by randomly inserting transposable DNA elements i.e. transposons into chromosomal DNA. When transposons were inserted into genes of interest, attenuation or a halt of expression occurred, thus showing that each of these genes of interest is crucial for CPS expression. Besides, another gene responsible for encoding an epimerase enzyme involved in capsule expression was also identified, and transposon insertion mutagenesis into this epimerase-coding gene indicated that mutants lost the ability to produce a discernible CPS (99). Plasmid mediated insertion of ORF9 DNA, having 85% homology with an epimerase produced by *V. cholerae* O139, into the chromosome of a non-encapsulated, translucent strain of *V. vulnificus*, completely restored CPS synthesis and reduced the LD₅₀ from 4.9×10^7 cells to only 9.7 cells (99). A group 1-like capsular polysaccharide operon for *V. vulnificus* was identified, and suggesting the function of the *wza* gene as an operon

essential for CPS synthesis in *V. vulnificus*. The *wza* gene is also a determinant for classification of Group I capsules (100).

The assembly system of capsules also influences virulence. Whitfield et al. (101) identified four distinct capsule assembly systems in *E. coli*, based solely on genetic and biosynthesis products that have since been used for classification of CPS in bacterial pathogens into Groups I-IV. Fifteen CPS types were identified among 38 clinical and environmental *V. vulnificus* strains, however two different serologically and sugar moiety distinct types were synthesized only by the more virulent clinical strains. They were later identified as Group I and Group IV capsules from sequencing and experimental mutation analyses of chromosomal DNA (28).

Iron uptake. Iron is essential for growth of microorganisms and plays an important role in bacterial virulence to human (102). In mammals, iron is intracellularly bound to lactoferrin (a form of transferrin protein) or as a cofactor in hemoglobin, and no free iron is available to invading bacteria so many pathogenic bacteria, including *V. vulnificus*, have developed various iron-scavenging mechanisms i.e. siderophores, hemophores and their receptors on the outer cell membrane as well as transferrin receptors in order to obtain iron from iron transport protein such as lactoferrin, and transferrin (103, 104). A recent publication from Skaar (104) proposes that “nutritional immunity” should be considered the first line of defense that literally starves bacterial pathogens of iron required for DNA replication needed for cell division and protection from oxidative stress. It has been shown that

Table 2. Gene clusters involved in synthesis of CPS in *V. vulnificus*

Functional Gene Clusters	Putative functions of CPS proteins
<i>Wcv</i> Cluster	
<i>wcvA</i>	Nucleotide-sugar epimerase (= an isomerase enzyme that catalyzes the rearrangement of –OH groups in biomolecules)
<i>wcvB</i>	Nucleotide sugar dehydrogenase
<i>wcvD</i>	Glycosyltransferase
<i>wcvE</i>	Glycosyltransferase
<i>wcvF</i>	Rhamnosyltransferase
<i>wcvG</i>	Glycosyltransferase
<i>wcvH</i>	Biosynthesis of activated monosaccharide precursors, confers serotype specificity
<i>wcvI</i>	Glycosyltransferase
<i>Rml</i> Cluster	
<i>rmlA</i>	Glucose-1-phosphate thymidyl-transferase, L-rhamnose synthesis
<i>rmlD</i>	dTDP-4-keto-L-rhamnose reductase, L-rhamnose synthesis
<i>rmlC</i>	dTDP-6-deoxy-D-xylo-4-hexulose 3,5 epimerase, L-rhamnose synthesis
<i>Wzy</i> Cluster	
<i>wecA</i>	Undecaprenyl phosphate N-acetyl glucosamine 1-phosphate transferase. Initiates transfer of activated repeat unit precursors such as GlcNa-1-phosphate to a phosphate lipid carrier.
<i>wza, wzb, wzc</i>	Translocation proteins that export CPS to outer cell surface, required for surface expression of Group I CPS. Wza forms a multimeric channel complex in outer membrane for polymer export, wzc is an inner membrane tyrosine autokinase, and wzb is the cognate phosphatase to wzc.
<i>wzx</i>	O-antigen flippase that translocates repeat sugar units across the periplasm. Also has been described as a polysaccharide translocase.
<i>wzy</i>	Wy-Polymerase and production of O antigen specific to Group IV capsules.

V. vulnificus can acquire iron from transferrin, hemoglobin, met-hemoglobin, and hematin and this ability is associated with virulence (76, 105-110). Siderophores are low molecular weight iron binding complexes that can bind atomic iron with equal or greater affinity than a host's iron sequestering transferrin or heme proteins. The presence of siderophore encoding genes (*vuuA*, *venB*, *vvsA* and *vvsB*) and their level of expression are correlated with degree of virulence of *V. vulnificus* (111-113). In addition to siderophore, Litwin et al. (111) showed that *V. vulnificus* is able to acquire non-transferrin bound iron with the help of heme receptor called Hup A, and also suggested that non-transferrin-bound iron is believed to be necessary for initiation of growth. There is evidence that the more virulent clinical strains can survive for longer periods in human serum than environmental strains due to their ability to acquire iron. Different survival period was observed among environmental and clinical *V. vulnificus* genotypes in human serum in terms of the significance of iron uptake, suggesting that the environmental strains (E type) are less able to survive compared to more-virulent C type strain (114). Proteases and hemolysin/cytolysin secreted by *V. vulnificus* is believed to aid in iron uptake through the breakdown of heme-binding proteins (115, 116)

Elevated iron in immune compromised and chronically ill individuals makes them more susceptible to *V. vulnificus* infection. In early studies of virulence, Wright et al. (117) determined that LD50 was much more reduced (from 10^6 to 1 CPU) in iron treated mice compared to untreated host. Two hypotheses of how excess iron gives an advantage to *V. vulnificus* have been proposed: 1) Excessive amount of iron is thought to result in decreased phagocyte activity (118), 2) Iron can significantly enhance the

replication and growth rate of *V. vulnificus*, thus resulting in increased susceptibility of iron-overloaded host to infection (119).

Pili. Attachment and colonization of host surface is important for host invasion by *V. vulnificus* because host cell contact is needed for cytotoxicity and virulence (113). Interaction of specific domain on a pilus with host cell surface receptor enables adherence of bacteria to host cells (120). In *V. vulnificus* strains, mutation of the *pilA* gene encoding pilin structural protein and *pilD* gene encoding for pre-pilin peptidase results in decreased adherence to epithelial cells and causes a slight reduction in virulence (31). Clinical strains of *V. vulnificus* collected from blood and wounds of infected individuals were found to have a higher number of pili per cell than environmental strains (121). The presence of pilus is obviously correlated with attachment, invasion of a host and virulence.

Flagella. Motility enabled by the presence of flagella also influences virulence. Mutations of genes *flgC*, encoding flagellar basal body, and *flgE*, encoding flagellar hook protein, resulted in defective motility, decreased attachment and cytotoxicity to HeLa cells that subsequently resulted in a decrease or loss of pathogenicity in *V. vulnificus* (32, 46). When *flgC* and *flgE* mutants were injected into mice to observe the effect of presence of flagella in virulence, the result showed that mutant strains had a higher LD₅₀ as compared to that of wild-type strains. The decrease in motility is also believed to diminish adhesion to host cells, which consequently causes loss of secretion

of cytotoxin and virulence so therefore production of flagella is considered a required factor for virulence (32, 46). It has also been suggested that in addition to their role in host cell adherence, flagella may serve as a type III secretion system for *V. vulnificus* toxins (32).

Outer membrane proteins. Pathogenic bacteria utilize outer membrane surface proteins for adhesion to and invasion of a host. Fibronectin is thought to be the most important host matrix for cellular adherence by pathogens (122). A fibronectin-binding protein in *V. vulnificus* (*ompU*) was determined by Goo et al. (33) to be a predominant protein in the outer membrane. Significant reductions in binding capacity to fibronectin was observed for mutant *ompU* strains as compared to wild-type strains, suggesting that defects in interaction between *ompU* and fibronectin results in decreased adherence to host cells at the start infection. This study also observed significant decreases in adherence of mutant *ompU* strains to HEP-2 cells as well as loss of cytotoxicity with ten-fold increases in LD₅₀, suggesting that cell-to-cell contact is required for cytotoxicity (33).

RtxA toxin. RtxA toxin has been identified as having an essential role in cytotoxicity of a broad range of pathogenic bacteria (123). In *V. vulnificus* its target activity is to depolymerize actin in host cells and its amino acid sequence and the organization of *rtx* gene cluster is highly similar (91% identical) to those in pathogenic strains of *V. cholerae* (124). Similar to the tests of virulence and CPS gene expression,

transposon mutagenesis attenuated expression of *rtxCA* and *rtxBDE* genes and decreased cytotoxicity toward INT-407 human intestinal epithelial cells (125). *RtxA* toxin produced by *V. vulnificus* is apparently only able to damage a host cell through cell-to-cell contact and therefore necessitates transfer to the outer cell membrane (113). Secretion of *RtxA* toxin is regulated by *rtxE* gene, on the *rtxBDE* operon. Transposon mutation of the *rtxE* gene eliminated the ability of *V. vulnificus* isolates to secrete *RtxA* toxin and resulted in decreased cytotoxicity (126).

Hemolysin/cytolysin. In addition to siderophores and hemophores, *V. vulnificus* produces hemolysin/cytolysin (*vvhA*) toxin that facilitates iron acquisition by lyses of hemoglobin (109, 115). Purified *vvhA* has also been found to increase vascular permeability, skin damage, and death rate in mice (35, 43). Moreover, the inoculation of hemolysin (*vvhA*) into mouse epithelial cells resulted in skin damage similar to those resulted from infection of *V. vulnificus* (35). *VvhA* can also stimulate production of guanylate cyclase in the host, causing an increase in the amount of intracellular cyclic GMP, and consequently vasodilation (127, 128). *VvhA* also functions as a “pore forming protein” in cellular membranes (129).

Metalloprotease. *V. vulnificus* is able to secrete an extracellular protease (*vvpE*) possessing collagenase, elastase and zinc-dependent metalloprotease activity (44, 130). Inoculation of purified *vvpE* into mice caused dermal necrosis, edema and increased vascular permeability (131). However, *vvpE* mutant strains did not show any reduction

in cytotoxicity or virulence in mice compared to wild-type strains (132). The metalloprotease component of *vvpE* is apparently not essential for virulence in *V. vulnificus* because no differences were found LD₅₀'s of mutant and wild type strains (133). Interestingly, an increase in the expression of *vvhA* (hemolysin-cytolysin) was observed in *vvpE* mutant strains suggesting that when the extracellular protease is absent, a greater amount of cytolysin is produced to sustain and even increase global virulence in *V. vulnificus* (133).

Biofilm and *V. vulnificus*

Due the nature of infections (i.e. septicemia and wound infections) the biofilm form in the life cycle of *V. vulnificus* has mostly been ignored. The few studies of biofilm formation by *V. vulnificus* have focused on the identification of proteins essential for biofilm development (pili, flagella, and outer membrane) (31, 46, 47, 134).

Bacterial biofilm is a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (135). Biofilm is a protective mode of growth of diverse range of organisms in both Archaea and Bacteria lineages in order to survive within hostile environments and disperse to nutrient rich environments for the establishment of new biofilm architectures (136). Bacteria in a biofilm exhibits distinct properties from their planktonic form (137). In general, biofilm forming microorganisms inherently represent more resistance to antimicrobials than planktonic counterparts (135, 137). Biofilm provides benefits to biofilm-forming cells in diverse harsh environments. Protections from desiccation, biocides, antimicrobial

treatments, host defenses, surfactants, and ultraviolet radiation are a few advantages of biofilm matrix integrity to colonized cells (138-140). Biofilm matrix also provides a suitable environment which facilitates the horizontal gene transfer between microorganisms, the sequestration of dissolved organic matters into the biofilm by the production of external digestive system, and allowing the use of those nutrients as energy sources (138).

The first step to form a biofilm is the process of bacterial attachment to inert or living surfaces (Fig. 1). The adhesion to inert surfaces is usually accomplished by nonspecific interactions (electrostatic, hydrophobic), while attachment to biotic surfaces i.e. living tissues is dictated by specific molecules such as lectin and other ligands (139). Microorganisms have to reach to an available surface in close proximity by either randomly flow of water or chemotaxis in order to loosely bind to surfaces, then irreversible binding takes place between surfaces and attached microbial cells by the production of exopolysaccharide (137). Pili and flagella are also involved in the first stages of biofilm formation. Flagella-mediated motility and the presence of pili favor the initial steps of biofilm formation and play a key role in biofilm formation in vibrios (51). After the irreversible attachment of microbial cells to surfaces, biofilm matures by proliferation and generation of extracellular dynamic polymeric matrix enveloping the attached microbial cells which are optimally structured to exploit the available nutrients (141, 142). Main matrix composition of a biofilm is comprised of microbial cells, water, exopolysaccharides and secreted /released extracellular products (142, 143). The matrix of biofilm is known as extracellular polymeric substance (EPS) which mediates

aggregation of the cells in close proximity to facilitate the complex interactions between bacterial cells and this EPS is also involved in mechanical stability and adhesion of individual cells in biofilm, thus facilitating the formation of multilayered architectural structure of biofilm (138, 141, 143). After maturation process reaches a critical mass, the dispersal of biofilm cells starts, in which the generated planktonic microorganisms escape the biofilm and disperse to new nutrient-rich environments for the establishment of new biofilm architectures (137).

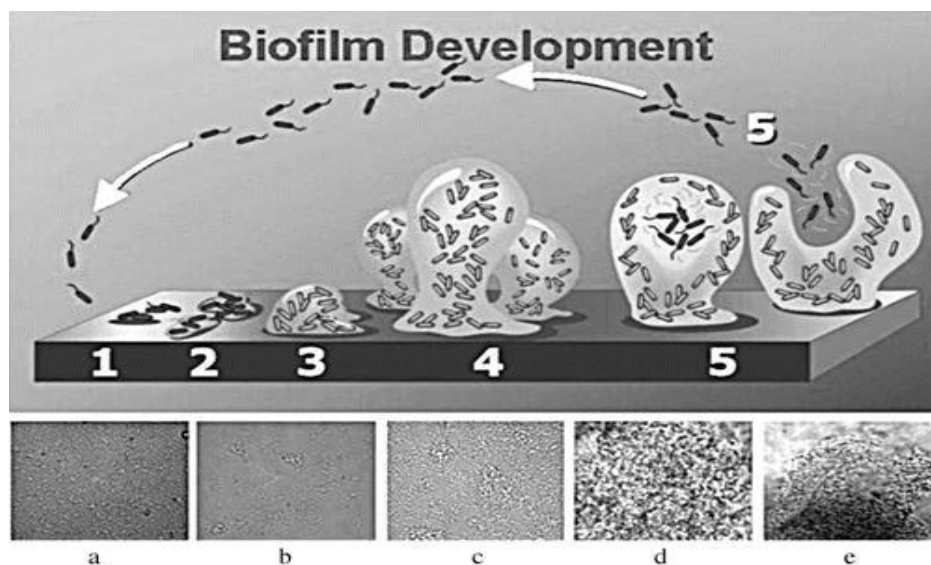


Figure 1: The five stages of biofilm development (144). Step1. reversible attachment; step2. irreversible attachment; step3. and step 4. maturation process; and step 5. dispersal.

Although the composition of biofilm matrix largely depends on intrinsic and extrinsic factors (142), *in vitro* experiments demonstrated that biofilm matrix generally consists of exopolysaccharides (EPS) (145, 146), structural proteins (138), extracellular enzymes (147, 148) (145, 148), extracellular DNA (146), lipids (149), and water (150). The combination of intrinsic and extrinsic factors generate a dynamic heterogeneous

microenvironment for biofilm-forming cells (143). EPS makes up an important proportion of matrix framework and is responsible for the attachment to various surfaces by its cohesive and adhesive property (138, 142). The mechanical stability and formation of biofilm architecture are provided mostly by the production of EPS (138), thus considered main structural component of biofilm matrix. Mutants defective in the production of EPS were disabled to form mature biofilms (47, 151). EPS are also the most major component of vibrio biofilms (51).

There are also several kinds of structural and enzymatic proteins involved in biofilm formation. The amount of protein in biofilm can remarkably differ between different biofilms. For example, the largest proportion (75%) of the *Pseudomonas putita* biofilm is comprised of proteins (152) although proteins typically make up 1-2% of most biofilm matrices (142). Structural proteins are responsible for the structural integrity and stability within biofilm (142). Accumulation-associated protein (Aap) in *Staphylococcus epidermis* is known to play a role in the establishment of intercellular adhesion (153). Additionally, Borlee et al. (154) identified a structural protein necessary for the contribution of structural integrity by exopolysaccharide cross-linking in *P. aeruginosa* biofilm.

There are several extracellular enzymes present in a mature biofilm. Various enzymes in biofilm are involved in the degradation of biopolymers and the utilization of other organic particles embedded into biofilms for carbon and energy source and some are responsible for virulence factors in medical biofilms (138). Extracellular DNA has also been considered as integral part of biofilm matrix (145, 155, 156) and serves as an

intercellular connector and is involved in stabilization of *P. aeruginosa* biofilms (157). It has been hypothesized that DNA contributes to the matrix network stability by the interaction of oppositely charged-matrix molecules (141, 156) .

It has been proposed that biofilm formation is a selective survival strategy of prokaryotic organisms to maintain their survival in harsh conditions, thereby facilitating the complex interactions, found only to be possible in close proximity between individual cells, and provides a suitable environment to communicate with each other by signaling pathways (136). Biofilm formation enables a microbial community capable of communicating each other via chemical signals called quorum sensing so it is one of the important factors for development of biofilm.

Physical and chemical variables such as temperature, pH, salinity, and iron concentration play important roles for the development of biofilms. Diaz Villanueva et al. (158) evaluated the interactive effects of temperature with respect to nutrient availability on biofilm development, hypothesizing that an increase in temperature would play more important role during the early stages of biofilm formation when cells in a thinner biofilm can be better exposed to nutrients. Results from this study also predicted that if the nutrients are the limiting factor for growth, temperature will show a greater effect on biofilms at higher nutrient levels. In other words, when nutrient availability regulates the biomass of mature biofilm, biofilm formation at higher temperature is faster. Townsley and Yildiz (159) tested biofilm formation by *V. cholerae* and found that biofilms produced at low temperatures (15°C and 25°C), like those experienced in the aquatic habitat, had increased biomass and thickness and were more

structured compared with those formed at 37°C. Another study (160) determined that *V. cholerae* produced slightly higher biofilm even at 30°C compared to 37°C. Di Bonaventura et al. (161) examined the effects of temperature and pH on biofilm development of 51 *Stenotrophomonas maltophilia* clinical isolates. They observed higher biofilm formation at 32°C than at either 18°C or at 37°C, and the amount of biofilm produced was significantly higher at pH 7.5 and pH 8.5 than at pH 5.5. This study also indicated that strain-to-strain differentiations, regardless of environmental factors, have a marked effect on the biofilm development of *S. maltophilia*. While most clinical strains of *S. maltophilia* produced biofilms, 2% of the clinical strains did not form biofilm under the same conditions. Seemingly, not all clinical strains have the potential to form biofilm, and environmental parameters and host factors are also important to enable the individual strains to form biofilm. *V. cholerae* produces biofilm for protection in the presence of bile acids (162) that normally restrict bacterial growth in the small intestine (163). Havasi et al. (164) found that one of the factors affecting the development of biofilm is sodium chloride, which might have a functional effect on biofilm formation and quorum sensing of *P. aeruginosa*. Iron regulates biofilm formation by several human pathogens (157, 165-167). *S. aureus*, that forms biofilms in chronic wounds (168) and is one of the most frequently isolated pathogens associated with nosocomial sepsis (169), produces more biofilm with increasing iron concentration, up to 200 µM (165). In some bacterial species, the concentration of iron is inversely correlated with the development of biofilm. Some studies have suggested that the increase in iron concentration causes a reduction in the amount of *P. aeruginosa* biofilm

(157, 170, 171). Other experiments with the cultures of *P. aeruginosa* have also shown that the increased concentration of iron, up to 100 μM FeCl_3 in polystyrene microtiter trays, suppressed the development of biofilm and subpopulation with decreased resistance toward antimicrobial compounds; conversely low iron concentration media (5 μM FeCl_3) favored the development of *P. aeruginosa* biofilm and of the resistance to antimicrobial compounds (157).

Swarming behavior in pathogenic bacteria has been correlated with biofilm formation in other pathogenic bacteria but has not yet been investigated in *V. vulnificus*. Thus far swarming behavior *V. vulnificus* has been tested for correlation with only a single virulence factor, hemolysin-cytolysin (*vvh*) production, and was determined to have either no or a down-regulatory effect (172). However, swarming behavior as well as production of vulnibactin, a siderophore, is both downregulated by the *LuxS* quorum sensing system and *Fur* (ferric uptake regulator) in *V. vulnificus* in the presence of iron rich media (173). Interestingly, Kim et al. (172) observed that swarming behavior was significantly increased when *V. vulnificus* was grown on softer 0.3% agar rather than the normal 0.5%. The semi-solid consistency of biofilms may be conducive to swarming in *V. vulnificus*. Swarming and quorum sensing may also be important strategies for host infection of wounds. If virulent clinical strains preferentially live attached to flocs of biofilm and extracellular polymeric substances in the water column, then the likelihood of wound infection encounter and successful adhesion may be higher than that for environmental strains living planktonically.

Adaptation of vibrios to fluctuating environmental factors is also dramatically important for their survival and colonization, suggesting that biofilm forming capacity is a fundamental factor for their survival in aquatic ecosystem (51). Studies with the sample of cholera patients revealed that biofilm-like aggregates were observed in those patients in addition to planktonic forms of *V. cholerae* (49). The biofilm forming ability of *V. cholerae* was examined under *in vivo* and *in vitro* conditions (49, 174, 175), and suggested that *V. cholerae* has the ability to form biofilm in both host and aquatic environments. Unlike *V. cholerae* host infections, *V. vulnificus* are not primarily restricted to the small intestine but instead occur in blood, skin, muscle, and other tissues, thus biofilm-forming ability of *V. vulnificus* in host phase has not yet been reported and/or rarely been investigated in clinical research due to the nature of infection. *In vitro* experiments reveal that *V. vulnificus* has the ability to develop biofilms (31, 45-48), so biofilm formation might be an important strategy for the survival of virulent strains versus less virulent environmental strains in marine environments until it can infect the host.

CHAPTER III

QUANTITATIVE PCR ENUMERATION OF *vcgC* AND 16S rRNA TYPE A/B GENES AS VIRULENCE INDICATORS FOR ENVIRONMENTAL AND CLINICAL STRAINS OF *VIBRIO VULNIFICUS* IN GALVESTON BAY OYSTERS

Overview

Oysters from a reef in Galveston Bay, TX, USA were screened for more virulent clinical versus less virulent environmental strains using a combination of quantitative PCR assays for the virulence correlating gene (clinical variant, *vcgC*) and 16S rRNA type A/B (type A = environmental, type B = clinical). The combination of *vcgC* and 16S rRNA type B loci to determine clinical type strains was suitable as indicated by the strong correlation ($R^2=0.98$, $p<0.001$) between these gene counts over time and their relative proportion (up to 93.8% and 94.3%, respectively) to *vvhA* genes used to quantify all strains of *V. vulnificus*. A strong seasonal shift of *V. vulnificus* strain types was observed. Environmental strains (16S rRNA type A) predominated from April to mid-June as salinities increased from 22 to 27 PSU and temperatures rose 20 to 28°C with peak gene quantities of $16,812\pm56$ CFU/g. As temperatures increased to $\geq 30^\circ\text{C}$ from mid-June to September and salinities rose above 27 PSU, clinical strains (16S rRNA type B; *vcgC*) predominated with peak quantities $31,868\pm287$ and $32,360\pm178$ CFU/g, respectively.

Introduction

V. vulnificus is a gram-negative, halophilic, autochthonous pathogen found in estuarine waters worldwide (176). Human infection typically occurs through ingestion of raw or undercooked seafood (177) but can also occur by contamination of wounds (13). *V. vulnificus* can cause gastroenteritis, necrotizing wound infections, and septicemia and is the leading cause of foodborne deaths in the USA (12). Although rare, *V. vulnificus* infections can be life threatening in predisposed individuals who have high serum iron levels and immune deficiencies (176). Death can occur within 24 hr after contact and mortality rates are greater than 50% in septicemic patients (176).

There are three known biotypes of *V. vulnificus*. Biotype 1 primarily infects humans, biotype 2 primarily infects eels, and biotype 3 is a hybrid of types 1 and 2 (12). Studies of *V. vulnificus* biotype 1 (hereafter referred to as simply *V. vulnificus*) have shown that there are degrees of pathogenicity or virulence. Historically, the more virulent strains were isolated from hospital patients and became known as ‘clinical’ strains whereas less virulent strains were isolated from seawater, oysters, fish, and other marine surfaces and became known as ‘environmental’ strains (23, 24). One of the first studies (23) to observe differences in virulence of clinical versus environmental strains used iron-overloaded mice and found that to achieve the same magnitude of infection as clinically isolated strains, environmentally isolated strains required a 350-fold higher inoculum. Since then focus has been on determining the variety of factors contributing to virulence. These include the extracellular capsule polysaccharide, endotoxins,

hemolysin/cytolysin, lipase, lecithinase, proteases, and collagenase as well as siderophores, pili, and flagella (37, 86, 97, 100, 178-180).

Bacterial genotyping studies have determined several polymorphic loci within the *V. vulnificus* genome that are indicative of clinical (C) versus environmental (E) strains. These loci include the virulence-correlated gene *vcg* (181, 182), 16S rRNA (25, 183), the siderophore-encoding *viuB* (vulnibactin) gene (40), and the capsular polysaccharide (CPS) operon (24). Several studies have used *vcg* to distinguish between clinical and environmental strains (39, 182, 184). *Vcg* has two sequence variants, *vcgC* and *vcgE*. Rosche (39) consistently detected the *vcgC* variant in clinical isolates (90%) whereas '*vcgE*' had a detection rate of 87% in environmental isolates. Another study however observed lower detection rates of *vcgC* (41%) and *vcgE* 59% (41). For 16S rRNA differentiation, sequence type B is associated with clinical strains and type A with environmental strains, however some environmental strains (mostly from oysters) have been found to possess both type B and type A sequences (12) and some type A strains have been isolated from hospital patients who recovered from infection (25). In a study of Louisiana oysters (41), 16S rRNA type B strains were detected 76% of the time in clinical isolates and type A only 15% of the time in environmental isolates. Greater agreement was observed by Nilsson et al. (25) who examined clinical isolates from patient fatalities (94% type B), patient recoveries (50% type B), and environmental strains from oysters or seawater (94% type A). The *viuB* gene, having one genotype, appears to be less specific with a 100% detection rate in clinical isolates but a 24% detection rate in environmental isolates (40). The CPS operon also does not differentiate

clinical versus environmental *V. vulnificus* with great accuracy. The CPS operon has two genotypes, 1 and 2. Genotype 1 matched clinical isolates 64% of the time and genotype 2 matched environmental isolates only 27% of the time (41). Han et al. (41) compared the accuracy of various combinations of loci. Regardless of whether two or three loci were used, combinations including *vcgC* and 16S rRNA type B gave higher percentages of clinical isolates (73.6 to 89.7%) while combinations including CPS and *viuB* gave lower percentages (56.2 to 66.5%). Although no single locus is 100% indicative of clinical versus environment strains, the virulence correlated gene *vcgC* and 16S rRNA type A/B appear to be the most promising thus far.

In this study we used quantitative PCR (qPCR) assays for the *vcgC* and 16S rRNA type A/B genes as well as the *vvhA* (*V. vulnificus*-specific hemolysin/cytolysin) gene to determine the occurrence and potential seasonality of clinical versus environmental strains of *V. vulnificus* in oysters. Thus far, genotyping studies of *V. vulnificus* virulence factors have primarily characterized clinical and environmental isolate cultures with traditional PCR. This study demonstrates the application of qPCR to oysters in their natural state. Quantitative PCR (qPCR) allows for rapid detection of targeted sequences without the requirement of time-intensive isolation and has been used to examine *V. vulnificus* in the environment and in experimental settings (78, 185, 186). Moreover qPCR can quantify cells that might be excluded during isolation methods.

Materials and methods

Sample collection and processing. Oyster samples harvested in 2009 and 2010 from a reef located in the west bay of Galveston Bay called ‘Sammy’s Reef’ (lat 29.254840° lon -94.918749°) were homogenized with a Waring blender (Fisher Scientific) and then diluted 1:10 (vol:vol) with alkaline peptone water (APW), incubated at 35°C with shaking at 100 rpm for 24 hours. A 1 ml aliquot was frozen in 2 ml tubes for later DNA extraction.

DNA extraction. Frozen samples were thawed and centrifuged in an AccuSpin Micro R Centrifuge (Fisher Scientific) at 4°C 10,000 rpm for 10 minutes. The supernatant was removed and 3% CTAB (cetyltrimethylammonium bromide, 500 µl) was added. The tubes were incubated for an hour in a water bath at 60°C. During incubation time, the tubes were vortexed every 10 minutes to facilitate the lysis of bacterial cell walls. Next, 500 µL of chilled 24:1 chloroform:isoamyl alcohol was added and vortexed on high for a good emulsion in order to dissolve the cell membrane of bacteria by binding lipids and proteins. The emulsions were centrifuged for 10 minutes at 10,000 rpm (4°C). At the end of the centrifugation a top aqueous layer containing DNA, a middle interphase with cellular debris, and a lower organic phase were visible. Lipids and proteins are non-aqueous compound and DNA/RNA is aqueous compound so DNA was in the aqueous phase while cell debris and organic phase were in the middle and lower organic phases. The aqueous phase was carefully transferred into a new 2ml Eppendorf tube and 2/3 volume of ice-cold isopropanol was added. Tubes were gently

tipped back and forward a few times to precipitate the DNA, and then stored at -20°C overnight. After overnight precipitation, the tubes were centrifuged 4°C 10,000 rpm for 10 minutes, the supernatant (isopropyl) was decanted and the tubes were air dried for 2 hr. Ice-cold ethanol (80%; 200 µl) was added to remove salts. The ethanol was decanted and the precipitated DNA was allowed to dry for 1-2 hours at room temperature with the tubes tipped downward and the caps off. DNA was rehydrated overnight at room temperature with a buffer containing 10 mM Tris, 10 mM NaCl, and 0.5 mM EDTA. DNA extracts were stored at -20°C.

Measurement of DNA concentration. The concentration of extracted DNA (ng/µL) was measured spectrophotometrically (NanoDrop ND-1000). The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA.

Multilocus qPCR assays. Four primers sets were used to quantify the virulence factor genes *vvhA*, 16S types A and B and *vcgC*. All reactions were performed in duplicate.

VvhA, the cytolysin-hemolysin gene that is specific to all *V. vulnificus*, was quantified in a SmartCycler (Cepheid) using gene specific primers and a probe labeled at the 5' end with Texas Red and at the 3' end with BHQ1 (Table 3). Thermal cycling was performed using a two-step PCR protocol: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The reaction mixture (20 µl) consisted of 1 µl template DNA (different concentrations), 1×PCR Buffer, 1 µM each primer, 250 nM

probe, 200 nM dNTPs, 2.5 mM MgCl₂, 1×BSA (bovine serum albumin), and 1 U Taq DNA polymerase (Roche). PCR positive control for qPCR and construction of standard curve was clinical strain 9038-96 obtained from the U.S. FDA Gulf Coast Seafood Laboratory.

16S A and *16S B* rRNA was quantified with a SmartCycler (Cepheid) using gene specific primers (Table 3) and Sybr Green intercalating dye. The reaction mixture (20 µl) consisted of 2 µl DNA template (different concentrations), 1 µM each primer, 2.5 mM MgCl₂, 1×BSA and 10 µl of IQ Sybr Green Supermix (BioRad) that contains Taq DNA polymerase, and dNTPs. Thermal cycling was performed using a three-step PCR protocol: 45 cycles of 95°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 25 s. The cycle threshold (*CT*) values were obtained when the fluorescence readings crossed a threshold of 30 units. Melt curve analysis was conducted from 60°C to 94°C at increments of 0.2°C per second. PCR positive controls for qPCR and construction of standard curves were clinical strain BUF7211 (type B) and environmental strain 98-640 (type A) obtained from the U.S. FDA Gulf Coast Seafood Laboratory.

VcgC (clinical variant) was quantified with a SmartCycler (Cepheid) using gene specific primers (Table 3) and Sybr Green intercalating dye. The reaction mixture (20 µl) consisted of 2 µl DNA template (different concentrations), 1 µM each primer, 2.5 mM MgCl₂, 1×BSA and 10 µl of IQ Sybr Green Supermix (BioRad) that contains Taq DNA polymerase, and dNTPs. Thermal cycling was performed using a three-step PCR protocol: 45 cycles of 95°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 25 s. The cycle threshold (*CT*) values were obtained when the fluorescence readings

crossed a threshold of 30 units. Melt curve analysis was conducted from 60°C to 94°C at increments of 0.2°C per second. PCR positive control for qPCR and construction of standard curve was clinical strain BUF7211 obtained from the U.S. FDA Gulf Coast Seafood Laboratory.

Standard curves were created in the Smart Cycler according to manufacturer instructions with the same primer sets and probes or intercalating dye using genomic DNA extracted from graduated concentrations (10^1 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , and 10^7) of control *V. vulnificus* strains containing the target gene. Counts (CFU) of *V. vulnificus* were generated by the standard curve and any dilutions were figured into the back calculation to the original DNA extraction volume. Final CFU/g was determined using the weight of the oyster tissue that was extracted.

Statistical Analysis. Regression analyses ($p>0.05$) and two sample T-test ($p>0.05$) were conducted with STATA 13.1 (StataCorp).

Table 3. Primers and probes used to quantify virulence factor genes in *V. vulnificus*.

Target Gene	Strain Specificity	Primer or Probe	Primer Sequence (5'-3')	References
<i>V. vulnificus</i> -specific hemolysin	all strains	VVH-F	TGTTTATGGTGAGAACGGTGAC	(78)
		VVH-R	TTCTTTATCTAGGCCCAAACTT	
		VVH-probe	Texas Red-CCGTTAACCGAACCA CCCGCAA-BHQ1	
<i>16S rRNA</i>	type A-environmental	VVAF1	CATGATAGCTTCGGCTCAA	(186)
		VVAR1	CAGCACTCCTTCCACCATCAC	
	type B-clinical	VVBF1	GCCTACGGGCCAAAGAGG	
VVBR1		GTCGCCTCTGCGTCCAC		
<i>vcgC</i>	clinical	VCGF3	CGCCTTTGTCAGTGTTGCA	(184)
		VCGB3	TAACGCGAGTAGTGAGCCG	

Results

Temperatures (Fig. 2) at Sammy's Reef did not rise above 20°C until 4/13/10 when they gradually increased to 31.5°C on 8/16/10. After 8/24/10 temperatures began to gradually decrease to below 20°C and then fell to 9°C on 12/13/10. Salinity (Fig. 1) hovered around 20 PSU until 4/13/10 and then gradually increased to 31 PSU on 6/8/10. There was a drop in salinity to 23-27 PSU in August with a spike in salinity on 9/4/10 and then gradual decrease to 15 PSU on 11/9/10 then increased steadily again to 32 PSU on 12/13/10.

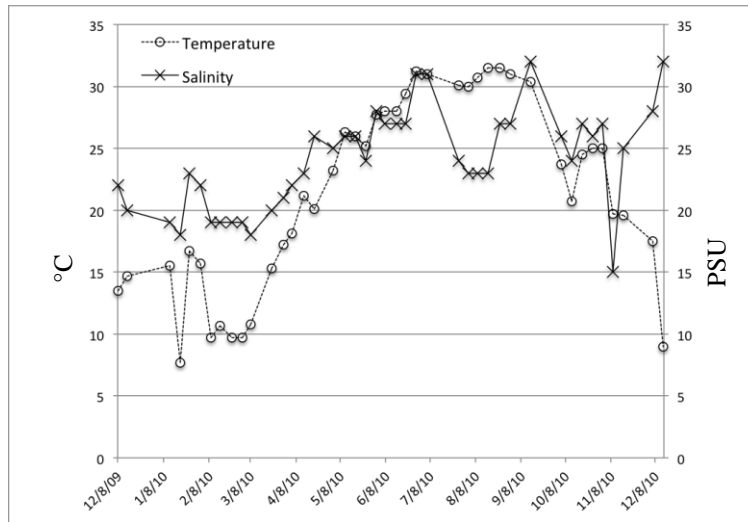


Figure 2. Temperature (°C) and salinity (PSU) in seawater at Sammy's Reef between 12/8/09 and 12/8/10.

VvhA (cytolysin-hemolysin) genes (expressed as CFU/g), used to quantify all strains of *V. vulnificus*, were undetectable from 12/8/09 to 4/13/10 with the exception of 2/9/10 where levels were 280 ± 8 CFU/g oyster homogenate and 3/2/10 with 200 ± 4 CFU/g (Fig. 3a) which were preceded by unusually warmer temperatures (22 and 23°C). *VvhA* counts rose steadily from $3,100 \pm 21$ to $34,153 \pm 110$ CFU/g between 4/13/10 and 8/31/10, with the rise in seawater temperature from 20 to $\geq 30^\circ\text{C}$. Between 9/14/10 and 11/16/10 *vvhA* counts decreased gradually from $32,249 \pm 132$ to $3,947 \pm 27$ CFU/g with the decrease in seawater temperature and were undetectable after 12/6/10 when temperatures dropped below 20°C.

16S rRNA type A genes, indicative of less virulent 'environmental' strains, were first detected on 2/9/10 and 3/2/10 (240 ± 9 and 196 ± 11 CFU/g, respectively) but then remained undetectable until 4/13/10 ($1,008 \pm 22$ CFU/g) when temperatures rose above

20°C and peaked on 6/15/10 with $16,812 \pm 56$ CFU/g (Fig. 3b). After 6/15/10, as temperatures rose to $\geq 30^\circ\text{C}$ quantities decreased gradually to $2,008 \pm 31$ CFU/g on 8/16/10 and remained at $< 2,000 \pm 27$ CFU/g until 11/16/10 and were non-detectable by December when temperatures dropped below 20°C.

16S rRNA type B genes, indicative of the more virulent ‘clinical’ strains were undetectable until 4/13/10 ($2,013 \pm 43$ CFU/g), but then increased steadily to $31,868 \pm 287$ CFU/g on 8/16/10 as temperatures reached $\geq 30^\circ\text{C}$ and then decreased gradually to $3,301 \pm 55$ CFU/g on 11/16/10 as temperatures dropped below 20°C (Fig. 3b).

VcgC, clinical variant, increased gradually from $1,750 \pm 29$ CFU/g on 4/13/10 to $32,360 \pm 178$ CFU/g on 8/31/10 then decreased gradually to $2,850 \pm 23$ CFU/g by 11/16/10 (Fig. 3c) and were non-detectable by December when temperatures dropped below 20°C.

Examination of proportion of 16S rRNA type A genes to the total *V. vulnificus* pool (i.e. % of *vvhA* genes) in oysters found that 16S rRNA type A genes could account for 32.5 to 74.5% of *V. vulnificus* from 4/13/10 to 6/28/10 (Fig 4a). After 6/28/10 proportion of 16S rRNA type A genes out of *vvhA* genes decreased to below 10%. Proportion of 16S rRNA type B genes to *vvhA* genes ranged from 79.8 to 93.8% between 8/3/10 to 11/16/10 (Fig. 4b). *VcgC* genes followed a similar distribution to 16S rRNA type B genes accounting for a high percentage of detected *V. vulnificus* (i.e. % of *vvhA* genes) ranging from 72.2 to 94.3% between 8/3/10 and 11/16/10 (Fig. 4c). This similarity was confirmed by a strong correlation ($R^2=0.98$, $p<0.001$) of *vcgC* to 16S rRNA type B gene counts (Fig. 5).

Discussion

We observed strong seasonality for 16S type B and *vcgC* genes indicative of clinical strains of *V. vulnificus* as well as for the 16S type A gene indicative of environmental strains in Sammy's Reef oysters. Environmental strains (i.e. 16S type A) dominated the pool of *V. vulnificus* from April to June as water temperatures gradually rose from 18 to 28°C and salinities increased from 22 to 27 PSU but were then succeeded by clinical strains (i.e. 16S rRNA type B and *vcgC*) from July through September when water temperatures were sustained at 30°C or above and salinities rose above 27 PSU. These results are in relative good agreement with Lin and Schwarz (53) who examined *V. vulnificus* isolates from Galveston Bay, TX, USA seawater over a 13 month period and found that 16S rRNA type A isolates were predominant from February to June as temperatures gradually rose to just above 20°C but then dropped dramatically when temperatures rose to between 25 and 30°C. 16S rRNA type B isolates predominated from July to October as temperatures rose to 30°C. Both 16S type A and B strains decreased rapidly with the onset of autumnal cold fronts and were non-detectable by

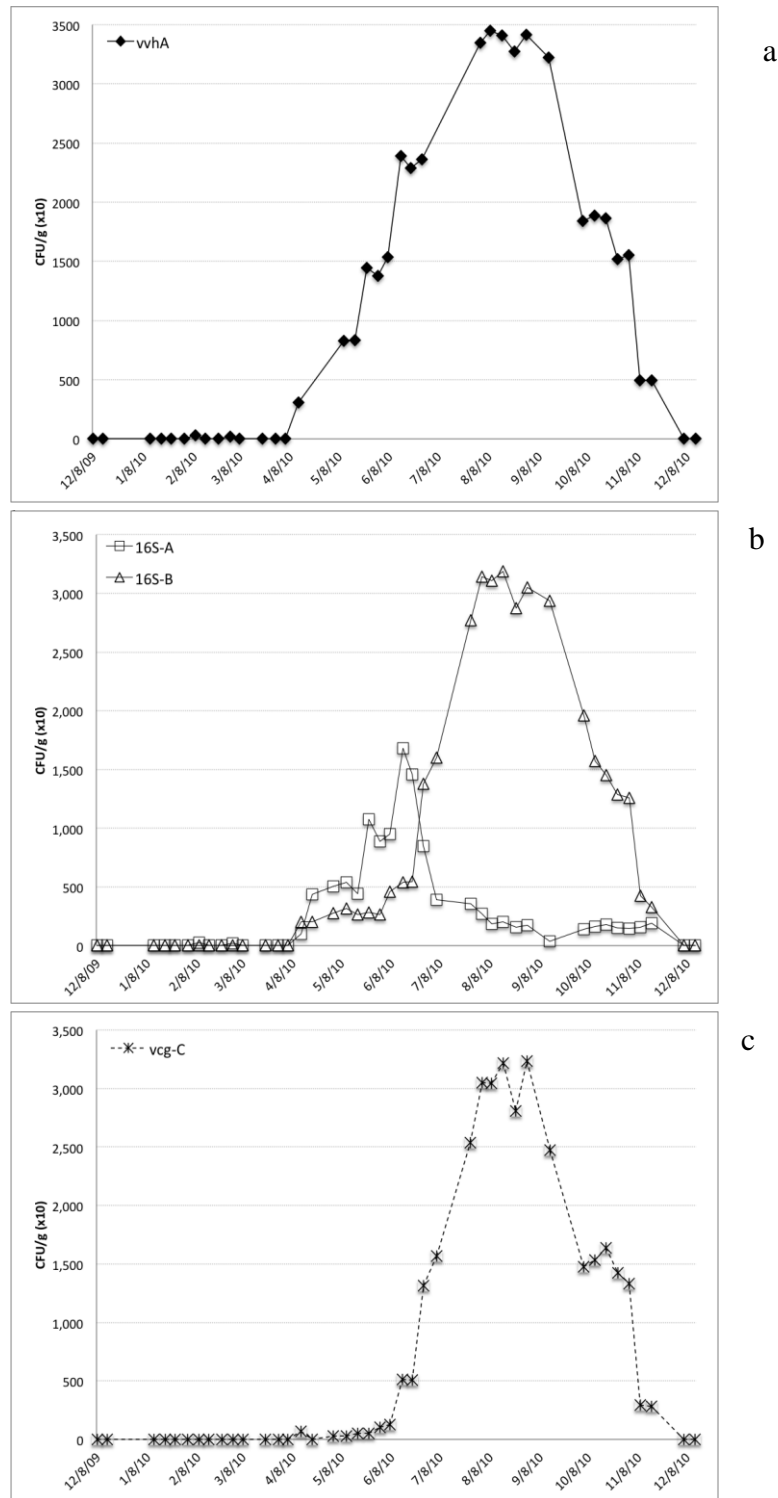


Figure 3. CFU/g a) *vvhA*, b) 16S rRNA types A and B, and c) *vcgC* in Sammy's Reef oysters between 12/8/09 and 12/8/10.

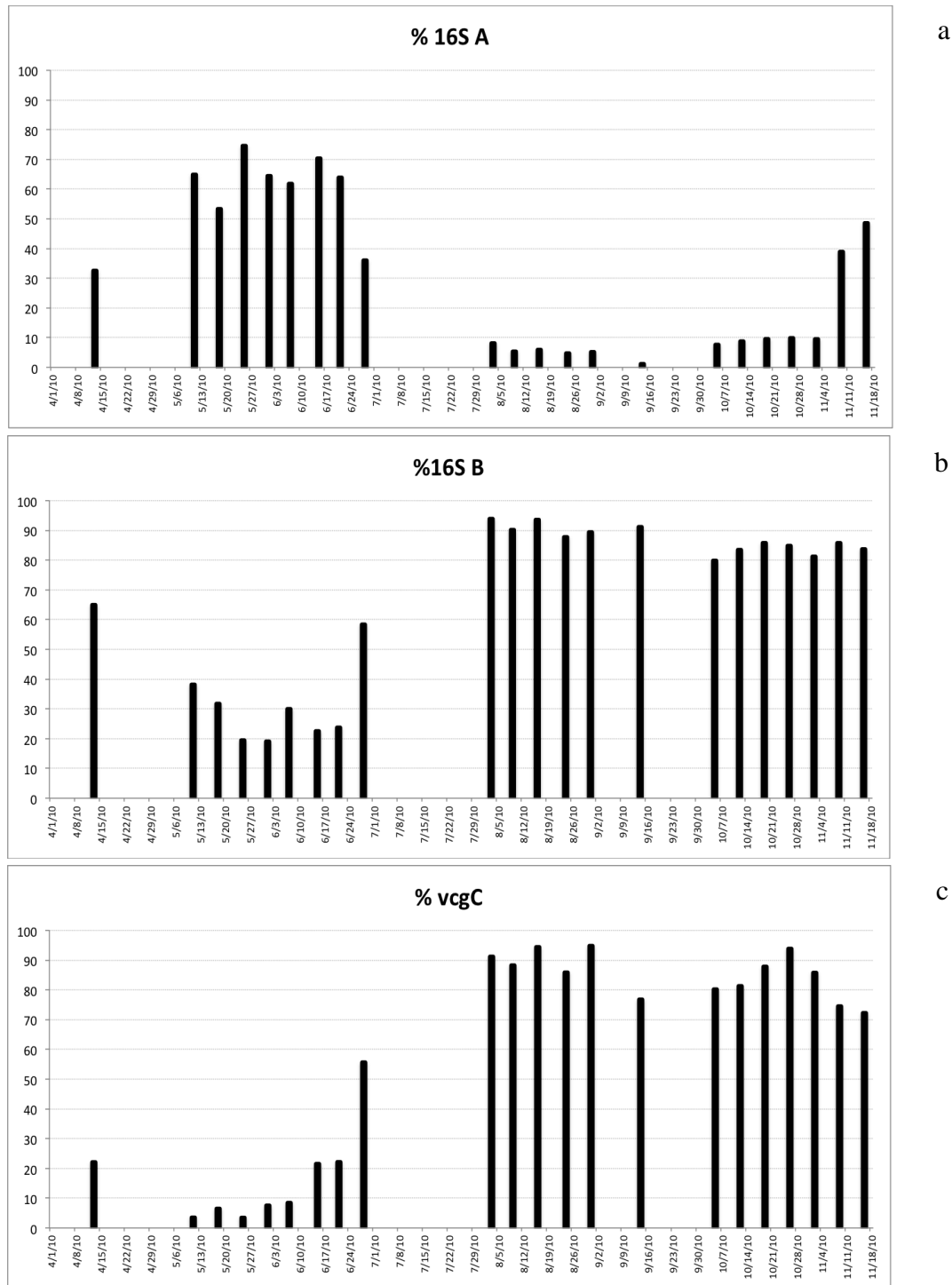


Figure 4. Proportion (%) of genes a) 16S rRNA type A, b) 16S rRNA type B, and c) *vcgC* out of the total *V. vulnificus* pool as determined by quantification of *vvhA* genes.

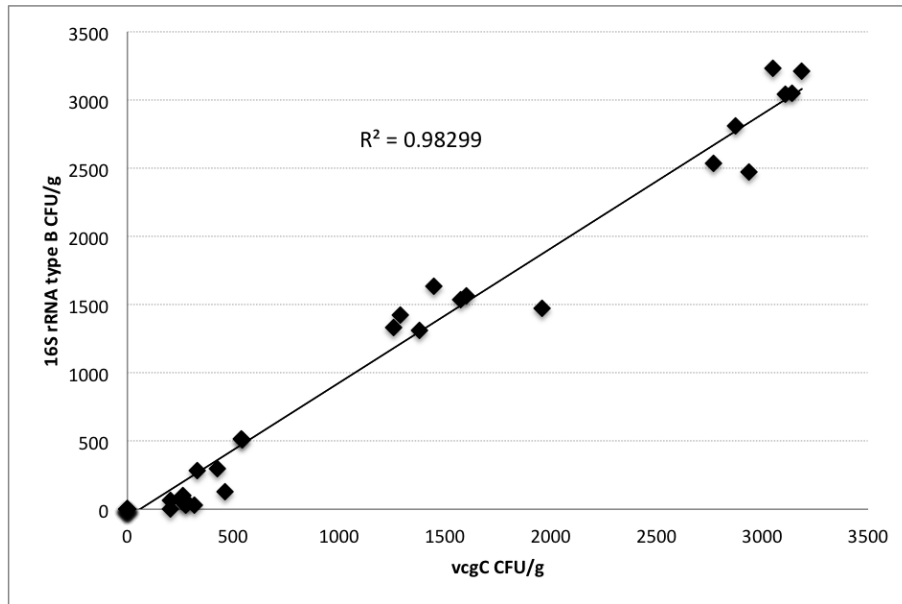


Figure 5. Correlation of 16S rRNA type B with *vcgC* CFU/g. $R^2=0.98$, $p<0.001$.

December. However, unlike the study by Lin and Schwarz (53) who isolated only 16S rRNA type A strains from February to May, we were able to detect both 16S rRNA type strains from April to December regardless of temperature or salinity. The difference was the quantities of one type to the other. In other words when 16S rRNA type A quantities were high, type B quantities were low and vice versa. This is likely a difference between occurrences of 16S rRNA types in seawater versus oysters that has been observed by other studies (182, 186).

Han et al. (41), characterized clinical versus environmental *V. vulnificus* isolates from Louisiana oysters using traditional PCR assays for 16s rRNA type A versus type B, and *vcgC* (clinical variant) versus *vcgE* (environmental variant). Similar to our findings, both 16S rRNA types could be isolated from oysters throughout the sampling period from April to September. A seasonal trend in predominance of one type versus the other

was also observed. 16S rRNA type B isolates increased in relative number in warmer months (July to September) 16S rRNA type A isolates were highest in relative number in cooler months (April to June). The same trend was observed for *vcgC* versus *vcgE* genes. Numbers of isolates having the clinical variant of *vcg* (*vcgC*) were predominant in July to September whereas the environmental variant of *vcg* (*vcgE*) was more prevalent from April to June. Lin and Schwarz (53) suggested that the difference in seasonal occurrence of 16S rRNA type A versus type B strains may be due the viability of each strain to be resuscitated from a 'viable but not culturable' state after extended periods of cold winter temperature. It would appear that the 16S rRNA type A strains have the ability to resuscitate sooner during cooler temperatures (around 10°C) and lower salinities (below 10 PSU) while 16S rRNA type B resuscitate only when temperatures reach 20°C and above and salinities rise to above 20 PSU. This seasonal difference is important for harvesting of oysters. Not only are total numbers of *V. vulnificus* lower in cooler months, environmental strains predominate thus lessening the likelihood of infection. However, some oyster reefs are harvested in warmer months. The predominance of clinical strains increases the likelihood of infection even if total numbers of *V. vulnificus* are low because fewer cells are required for infection due to greater virulence.

We observed a strong correlation of 16S rRNA type B and *vcgC* genes in this study ($R^2=0.98$; $p<0.001$; Fig. 5) as did Han et al. (41) ($R^2=0.99$; $p<0.001$). Han et al. (41) also examined other virulence factors such as allele type (clinical versus environmental for the capsular polysaccharide operon) and prevalence of the *viuB*

(siderophore encoding) gene as an indicator of clinical strains. Neither of these factors showed a strong correlation to occurrence of *vcgC* ($R^2=0.68$, $p=0.023$ and 0.48 , $p=0.124$, respectively) or to 16S rRNA type B ($R^2=0.68$, $p=0.043$ and 0.45 , $p=0.139$, respectively). Conversely, other researchers have proposed that 16S rRNA type may not be suitable for distinguishing clinical versus environmental strains. Gordon et al. (186) found that some environmentally isolated strains from oysters possessed both 16S rRNA gene types (A and B) whereas clinically isolated strains only possessed 16S rRNA type B genes. The overlap of 16S rRNA types in oysters determined in our study may be explained by presence of strains possessing both types (AB). Indeed, between 4/13/10 and 6/7/10 the occurrence of type B genes was significantly higher (t-test, $p = 0.000$) than *vcgC* genes and distribution of the two had low correlation ($R^2=0.37$; $p=0.105$). This may be explained by a preponderance of type AB strains, but we did not confirm this with isolate cultures. However as water temperatures rose to 30°C and above the correlation of *vcgC* and 16S rRNA type B genes was strongest ($R^2=0.99$; $p<0.001$).

Data on the correlation of genotype with *in vivo* testing of virulence are limited but are still useful in confirming the suitability of the *vcgC* and 16S rRNA type A/B loci to differentiate between clinical and environmental strains. Thiaville et al. (26) who examined the relationship of genotype to virulence in subcutaneously inoculated, iron dextran-treated mice found that 16S type A and AB strains had significantly lower incidence of skin and liver infection whereas type B had significantly higher incidence. Moreover, death rate was significantly higher for type B and AB strains than type A. This study also found that incidence and magnitude of skin infections was not different

for *vcgE* and *vcgC* genotypes however *vcgC* strain-infected mice had a significantly rates of systemic infection and death. Nilsson et al. (25) also observed differences of virulence in hospital patients. Isolates from patients who died were consistently type B (94%) but isolates from patients who recovered were 50% type B and 50% type A. Although there appears to be degrees of virulence within 16S rRNA type B, the use of assays for 16s rRNA A/B to differentiate clinical versus environmental strains are still useful in understanding the ecology of *V. vulnificus*.

The results of our study confirm that are seasonal shifts in the virulence of the *V. vulnificus* population in oysters as demonstrated by the dominance of less virulent environmental strains in cooler months and more virulent clinical strains in warmer months. We found that the combination of *vcgC* and 16S rRNA A/B loci to be highly suitable differentiation of these strains. A comparison of 16S rRNA type A+B gene counts to *vvhA* gene counts (i.e. total *V. vulnificus*) gave an 83 to 102% agreement indicating that most of the *V. vulnificus* pool had been detected by qPCR. A similar comparison of *vcgC* gene counts to *vvhA* gene counts gave a 55 to 95% coverage with the majority being >80%. However, the caveat of qPCR is that it also detects dead cells so that the data may not a true representation of the viable population. The combined application of these qPCR assays to rapidly screen oysters and other seafood for more virulent clinical strains of *V. vulnificus* will eliminate the need for lengthy isolation methods.

CHAPTER IV

SELECTIVE ENRICHMENT FOR CLINICAL STRAINS OF *VIBRIO VULNIFICUS*

Overview

Traditional enrichment and maintenance media for *V. vulnificus* were tested for selective preference for clinical or environmental strains. In the laboratory, Alkaline Peptone Water (APW) and Brain Heart Infusion Broth (BHIB), with adjusted salinity to that of APW, were inoculated with equal concentrations of clinical and environmental strains and incubated for 20 hr at 35°C. BHIB selected for clinical strains (85%) as indicated by percentage of *vcgC* to *vvhA* genes, enumerated with quantitative PCR. In APW, the ratio of clinical to environmental strains was roughly equal. Enrichments of seawater with BHIB, APW, and Luria Bertani broth (LB) resulted in higher % clinical strains in BHIB but not in APW or LB, in most samples. However seasonal differences in predominance of clinical or environmental strains in the seawater samples influenced the enrichment process. The selective preference of the BHIB10 medium for clinical strains indicates that iron plays an important role in the infection of the human host.

Introduction

Life-threatening, halophilic, gram-negative marine bacterium *Vibrio vulnificus* was first isolated by the Centers for Disease Control (CDC) in 1964 (68), since then, tremendous threats to human health as well as seafood industry have been documented (12, 68). This opportunistic human pathogen, among the most severe of all foodborne

infections, causes primary septicemia and severe necrotizing wound infections with a mortality rate exceeding 50%, mostly in susceptible individuals having an underlying disease such as hemochromatosis, immune disorders, and diabetes. Infection is mainly caused by ingestion of raw or undercooked oysters but can also be the result of exposure of pre-existing wounds to seawater or seafood products (68, 187). Death occurs in one or two days after the onset of disease unless an effective treatment can be implemented (68, 188).

V. vulnificus biotype 1, primarily infecting humans, has been demonstrated to have degrees of virulence. Strains isolated from hospital patients, referred to as ‘clinical,’ are highly virulent, whereas strains isolated from seawater, oysters, fish, and sediments, referred to as ‘environmental,’ are typically less virulent with some exceptions (23, 68, 189-193). Human tissue appears to select for more virulent clinical strains of *V. vulnificus* while those isolated from oysters exhibit a high level of virulence diversity (194). Virulence is conferred by several factors including the extracellular capsule polysaccharide (CPS), hemolysin/cytolysin, lipase, lecithinase, proteases, and collagenase as well as pili, and a flagellum (12, 37, 86, 97, 100, 178, 180). Capsular polysaccharide (CPS) is considered to play an important role in virulence of *Vibrio vulnificus* and its expression associated with the degree of virulence was shown in animal models because CPS enables microorganism to evade host immune response by avoiding phagocytosis and complement mediated immunity (87, 195).

Hemolysin/cytolysin is responsible for lysis of host erythrocytes and the subsequent release of iron, in turn, stimulates transcription of the *vvhA* gene (196) and is required for

the efficient transcription of the extracellular protease VvpE. Extracellular enzymes play an important role in tissue necrosis (12). Pili and flagella are used for attachment to host cells and formation of biofilms (12).

Most studies of the virulence of *V. vulnificus* have relied upon isolate cultures. In the clinical environment *V. vulnificus* from tissues and blood are isolated on blood agars (69). For environmental samples, isolation methods involve an overnight enrichment in alkaline peptone water (APW), having a salinity of 10 PSU, that selects for halophilic bacteria (197). Enrichment is followed by streaking onto differential-selective agars. For several decades, thiosulfate-citrate-bile salts-sucrose (TCBS) agar was used to select for and isolate pathogenic vibrio species (70). Superior differential-selective media agars specific for *V. vulnificus* have since been developed and are now standard method. The first, cellobiose-polymyxin B-colistin (CPC) agar utilizes the resistance of *V. vulnificus* to colistin and polymyxin B for selection (198). Other standard agars are modifications of CPC. Tamplin et al. (75) modified CPC (mCPC) by reducing the amount of colistin to improve isolation success from environmental samples. Høi et al. (199) was able to significantly increase the isolation rates of *V. vulnificus* from seawater and sediments by removing polymyxin B from CPC to make CC agar.

Because of the discovery of clinical and environmental strains it is important to know if standard methods for isolation (i.e. enrichment in liquid media followed by selective/differential agars) select for one strain over the other. Warner and Oliver (200) determined through genotyping of the virulence correlating gene (*vcgC*) that CPC and mCPC agars select preferentially for clinical strains. We were interested in knowing if

the prior overnight enrichment step with liquid media could preferentially select for clinical versus environmental strains of *V. vulnificus*. We compared APW, the traditional enrichment broth for *V. vulnificus* and other vibrios (201), to media employed for maintenance of *V. vulnificus* isolate cultures in the laboratory such as brain heart infusion broth (BHIB) (39, 181), and Luria Bertani broth (19, 78, 202).

We hypothesized that different enrichment media will influence the selection for *V. vulnificus* clinical and environmental strains. Furthermore, we hypothesized that BHIB, which contains whole cells of calf brains and heart and has a higher iron concentration (24.5 μM) than other media we tested ($\sim 7 \mu\text{M}$), is most like human blood that selects for more virulent clinical strains. Normal iron concentrations of human serum are 14-32 μM in males and 10-28 μM in females and in persons with hemochromatosis 40 to 50 μM (203, 204). And, we wanted to know if the standard enrichment media APW is selective and would consequently influence the ratio of environmental versus clinical strains on differential-selective agars.

Since standard isolation methods for *V. vulnificus* utilizes agars (i.e. CPC and mCPC) that select for clinical strains, we opted to use quantitative PCR (qPCR) assays to detect clinical strains directly in the enrichment media. QPCR allows for rapid detection of targeted sequences without the requirement of time-intensive isolation and has been used to examine *V. vulnificus* in the environment and in experimental settings (78, 185, 186). Moreover qPCR can quantify cells that might be excluded during isolation methods. We quantified the hemolysin/cytolysin gene, *vvhA* that is present in all *V. vulnificus* for total cell counts. Panicker et al. (84), who developed the assay, found

it to have absolute specificity for *V. vulnificus* and high correlation ($R^2=0.98$) with plate counts. The clinical variant of the virulence correlated gene (*vcgC*) was used to quantify clinical strains of *V. vulnificus* (184). The *vcgC* gene clinical variant has a 90% detection rate in clinical isolates (39). Two studies observed a high correlation ($R^2=0.98$; $R^2=0.99$, respectively) with the 16S rRNA type B gene (41) that is also present in clinical isolates.

Materials and methods

Isolate enrichments. This experiment was designed to test if clinical or environmental isolates were preferentially selected in enrichment media. Ten ml (3 replicates) of APW broth having a salinity of 10 PSU ('APW10') and BHIB with an adjusted salinity of 10 PSU ('BHIB10') were inoculated with equal volumes (10 μ l) of log phase ($OD_{600\text{ nm}} = 0.6$) clinical (BUF7211) and environmental (98-640) strains of *V. vulnificus* (obtained from the U.S. FDA Gulf Coast Seafood Laboratory) and incubated for 20 hr at 35°C with shaking at 100 rpm. After incubation, a 2 ml aliquot was transferred into 2 ml tubes, centrifuged for 10 min at 10,000 rpm (4°C) to obtain a bacterial cell pellet and the supernatant was removed.

Seawater enrichments. This experiment was designed to determine if enrichment media preferentially enriched clinical versus environmental *V. vulnificus* from environmental samples. Five ml (3 replicates) of APW having a salinity of 10 PSU, APW with 1/2 concentration of NaCl ('APW5'; 5 PSU), BHIB ('BHIB5'; 5 PSU), BHIB modified to a salinity of 10 PSU, and Luria Bertani broth ('LB10'; 10 PSU), were

inoculated with five ml of seawater, collected from six locations in Galveston Bay (GB4 lat 29.576801°/lon -94.934458°; GB6 lat 29.407004°; lon -94.806573°; GB12 lat 29.478671°/lon -94.757890°; GB17 lat 29.231857°/lon -94.989873°; GB25 lat 29.697525°/lon -94.783926°; GB29 lat 29.649323°/lon -94.811337°) in March, September, and November 2012 and vortexed then incubated for 20 hr at 35°C with shaking at 100 rpm. After incubation, a 2 ml aliquot was transferred into 2 ml tubes, centrifuged for 10 min at 10,000 rpm (4°C) to obtain a bacterial cell pellet and the supernatant was removed.

DNA extraction. CTAB (3% cetyltrimethylammonium bromide, 500 µl) was added to the pellets. The tubes were incubated for an hour in a water bath at 60°C. During incubation time, the tubes were vortexed every 10 minutes to facilitate the lysis of bacterial cell walls. Next, 500 µl of chilled 24:1 chloroform:isoamyl alcohol was added and vortexed on high for a good emulsion in order to dissolve the cell membrane of bacteria by binding lipids and proteins. The emulsions were centrifuged for 10 minutes at 10,000 rpm (4°C). At the end of the centrifugation a top aqueous layer containing DNA, a middle interphase with cellular debris, and a lower organic phase were visible. Lipids and proteins are non-aqueous compound and DNA/RNA is aqueous compound so DNA was in the aqueous phase while cell debris and organic phase were in the middle and lower organic phases. The aqueous phase was carefully transferred into a new 2ml tube and 2/3 volume of ice-cold isopropanol was added. Tubes were gently tipped back and forward a few times to precipitate the DNA, and then stored at -20°C

overnight. After overnight precipitation, the tubes were centrifuged 4°C 10,000 rpm for 10 minutes, the supernatant (isopropyl) was decanted and the tubes were air dried for 2 hr. Ice-cold ethanol (80%; 200 µl) was added to remove salts. The ethanol was decanted and the precipitated DNA was allowed to dry for 1-2 hours at room temperature with the tubes tipped downward and the caps off. DNA was rehydrated overnight at room temperature with a buffer containing 10 mM Tris, 10 mM NaCl, and 0.5 mM EDTA. DNA extracts were stored at -20°C.

Quantitative PCR. To determine total *V. vulnificus*, we quantified *V. vulnificus* specific hemolysin (*VvhA*) genes with a SYBR Green quantitative PCR (qPCR) assay (84). Total volume was 25 µL which contained 12.5 µL SYBR green Supermix (BioRad), 1 µM *vvh-L* primer (5'-TTCCAACCTCAAACCGAACTATGA-3'), 1 µM *vvh-R* primer (5'-ATTCCAGTCGATGCGAATA CGTTG-3'), 2.5 mM MgCl₂, 1 X BSA, and 2 µL of template DNA. The PCR reaction was performed in a SmartCycler (Cepheid) at 94°C for 2 minutes, followed by 45 cycles of at 94°C for 15 seconds, at 56°C for 15 seconds and at 72°C for 25 seconds. Melt curve analysis was conducted from 60°C to 94°C at increments of 0.2°C per second. PCR positive control for qPCR runs and construction of standard curve was clinical strain BUF7211 obtained from the U.S. FDA Gulf Coast Seafood Laboratory.

To determine clinical strains, we used a SYBR Green qPCR assay that quantifies the clinical variant of the virulence correlating gene (*vvg*) (205). Total volume was 20 µL which contained 10 µL SYBR green Supermix (BioRad), 1 µM of each primer;

VCGF3 (5'-CGCCTTTGTCAGTGTGCA-3') and VCGB3 (5'-TAACGCGAGTAGTGAGCCG-3'), 2.5 mM MgCl₂, 1 X BSA, and 2 µL of DNA template. The PCR reaction was performed in a SmartCycler (Cepheid) at 95°C for 2 minutes for initial denaturation, followed by 45 cycles at 95°C for 20 seconds and 60°C for 30 seconds and 72°C for 25 seconds. Melt curve analysis was conducted from 60°C to 94°C at increments of 0.2°C per second. PCR positive control for qPCR and for construction of standard curve was clinical strain BUF7211 obtained from the U.S. FDA Gulf Coast Seafood Laboratory.

Standard curves were created in the Smart Cycler according to manufacturer instructions with the same primer sets using genomic DNA extracted from graduated concentrations (10¹, 10², 10³, 10⁴, 10⁵, 10⁶, and 10⁷) of *V. vulnificus* strain BUF7211. Counts (CFU) of *V. vulnificus* were generated by the standard curve and any dilutions were figured into the back calculation to the original DNA extraction volume.

Two sample T-test and One-way ANOVA ($p < 0.05$) was conducted with STATA 13.1 (StataCorp). For ANOVA Bartlett's test was conducted *a priori* to ensure equal variances. Bonferroni *post hoc* test was used to determine differences in means ($p < 0.05$).

Results

Isolate enrichments. *VvhA* concentration, as a proxy for *V. vulnificus* cells, was the same in enrichment media APW10 ($1.5 \times 10^6 \pm 3.7 \times 10^5$ CFU/ml) and BHIB10 ($1.6 \times 10^6 \pm 3.6 \times 10^5$) (Fig. 4-1) (two-sample T-test; $p = 0.8695$). Percent *vcgC* genes was

higher in the BHIB10 medium ($85.6 \pm 3.4\%$) than in the APW10 medium ($47.3 \pm 7.6\%$) (Two-sample T-test; $p=0.0234$) (Fig. 6).

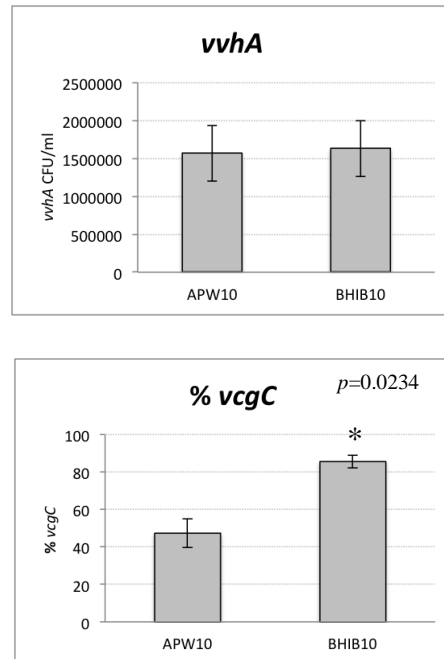


Figure 6. Concentration of *vvhA* (CFU/ml) and *% vcgC* genes in enrichment media APW10 and BHIB10. Asterisk denotes significant difference $p < 0.05$.

Seawater enrichments. *VvhA* concentrations (CFU/ml) are presented in Fig. 7 according to sampling date and station numbers. For samples collected on 3/22/12, no significant differences were observed between enrichments in samples GB12 and GB25 (ANOVA; $p=0.748$, $p=0.245$, respectively). Concentrations in sample GB17 enrichments APW5 and BHIB5 were significantly lower than APW10, BHIB10, and LB10 (ANOVA; $p=0.0097$). Total *V. vulnificus* (i.e. *vvhA* CFU/ml) in all samples ranged from 76 ± 11 to 275 ± 44 CFU/ml.

For sample GB25, collected on 9/4/12, *vvhA* concentrations in enrichments APW10 ($1,335 \pm 133$ CFU/ml), BHIB10 ($1,469 \pm 376$ CFU/ml) and LB10 ($1,026 \pm 145$ CFU/ml) were not significantly different from each other however they were approximately 4 to 6 fold higher (ANOVA; $p=0.0026$) than the other enrichments with APW5 and BHIB5. Enrichments APW10 ($1,747 \pm 134$ CFU/ml) and BHIB10 ($1,920 \pm 158$) in sample GB29, collected on the same day, had significantly higher *vvhA* concentrations than all other enrichments (ANOVA; $p=0.0001$). And enrichment LB10 ($1,076 \pm 81$) had significantly higher concentrations than APW5 (435 ± 32 CFU/ml) and BHIB5 (422 ± 92 CFU/ml) (Bonferroni *post hoc* test; $p=0.020$ and $p=0.018$, respectively)

For samples collected on 11/4/12, GB4, GB6, and GB12, *vvhA* concentrations in the APW10 (183 ± 23 , 335 ± 66 , 478 ± 45 CFU/ml, respectively), BHIB10 (150 ± 10 , 368 ± 57 , 324 ± 46 CFU/ml, respectively), and LB10 (132 ± 14 , 242 ± 27 , and 321 ± 13 CFU/ml, respectively) enrichments were not significantly different from each other but were approximately 3 to 4 fold higher ($p=0.0002$, $p=0.0107$, and $p=0.0008$, respectively) than the other enrichments, APW5 and BHIB5.

The percentage of *vcgC* gene counts out of total *V. vulnificus* cell counts (i.e. *vvhA*) are presented in Fig. 8 according to sampling date and station numbers. All samples collected on 3/22/12 had low percentages (<20%) of the *vcgC* gene with no significant differences between enrichments.

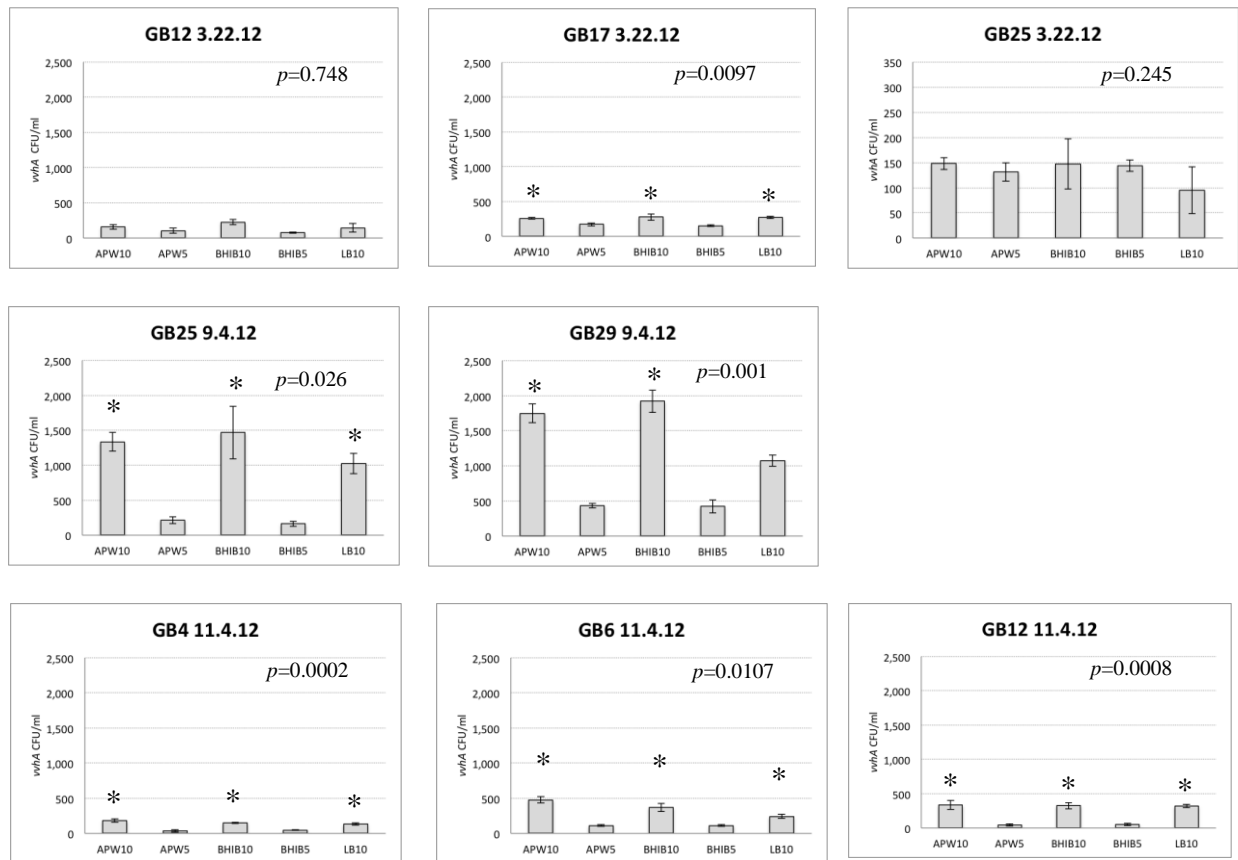


Figure 7. *VhA* CFU/ml in seawater enriched with APW10, APW5, BHIB10, BHIB5, and LB10 for samples collected 3/22/12, 9/4/12, and 11/4/12 analyzed with one-way ANOVA, $p < 0.05$. Asterisk denotes significance of $p < 0.05$ in Bonferroni *post hoc* test.

Sample GB25 collected on 9/4/12 had significantly higher *vcgC* percentages in enrichment BHIB10 (68 ± 6 ; $p < 0.01$ Bonferroni *post hoc* test) than APW10 and LB10 (49 ± 6 and $33 \pm 9\%$, respectively), that were higher ($p < 0.05$, Bonferroni *post hoc* test) than the other enrichments having $\sim 10\%$ (ANOVA; $p = 0.0006$). Percent *vcgC* genes in the BHIB10 enrichment ($70 \pm 3\%$; $p < 0.01$ Bonferroni *post hoc* test) from sample GB29 was significantly greater than all other enrichments APW10 ($52 \pm 3\%$), APW5 ($9 \pm 3\%$), BHIB5 ($8 \pm 2\%$) and LB10 ($46 \pm 2\%$) (ANOVA; $p < 0.0001$).

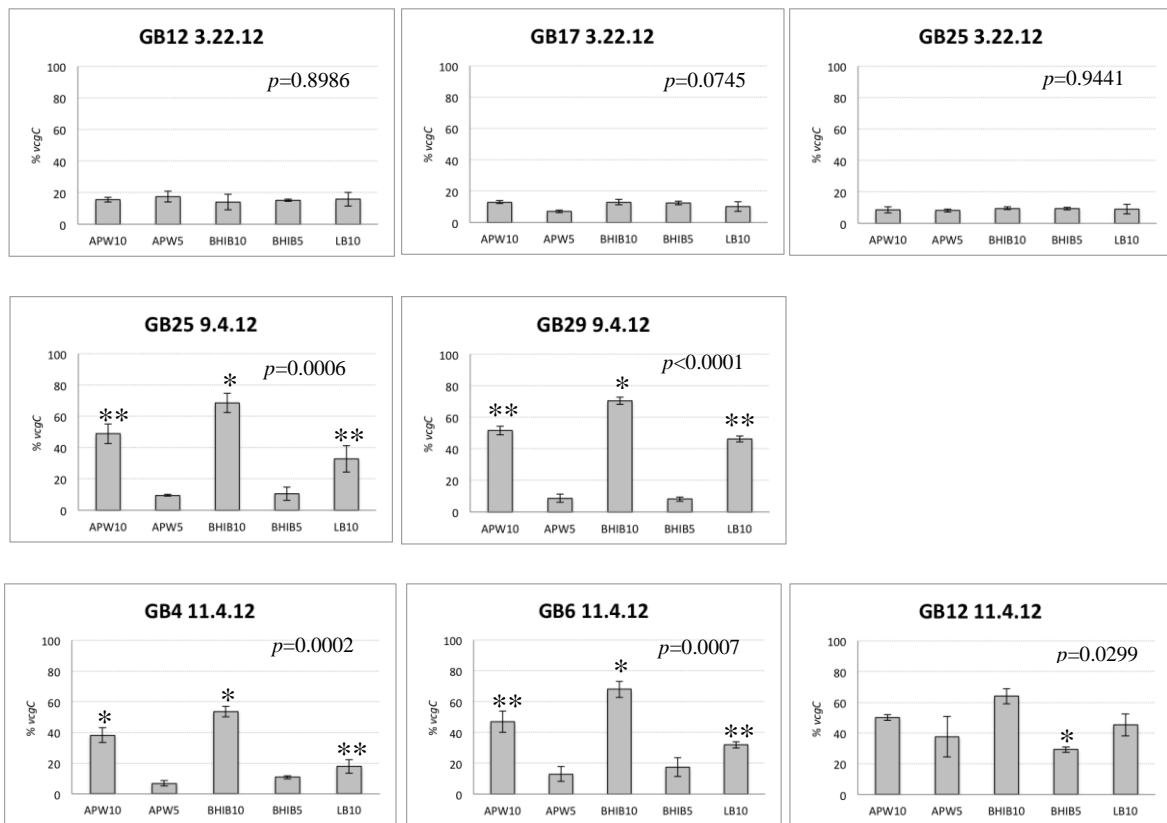


Figure 8. Percent *vgcC* in seawater enriched with APW10, APW5, BHIB10, BHIB5, and LB10 for samples collected 3/22/12, 9/4/12, and 11/4/12 analyzed with one-way ANOVA, $p<0.05$. Asterisk denotes significance of $p<0.01$ and double asterisk $p<0.05$ in Bonferroni *post hoc* test.

Percent *vgcC* genes in enrichment BHIB5 from sample GB12 collected on 11/4/12 was significantly lower (ANOVA $p=0.03$) than the other enrichments, which were equal to each other ($p<0.05$ Bonferroni *post hoc* test). While in samples GB4 and GB6, no differences in % *vgcC* was determined between enrichments APW10 and BHIB10 ($p<0.01$ Bonferroni *post hoc* test), but they were significantly greater than LB10 ($p<0.05$) that was higher than APW5 and BHIB5 (ANOVA; $p=0.0002$ and $p=0.0007$, respectively).

Discussion

As hypothesized, the BHIB10 medium selected for clinical strains of *V. vulnificus* likely due to the high iron content. This selection could be advantageous for studying virulence factors in *V. vulnificus*. The ratio of clinical to environmental strains in APW10 was roughly equal indicating that it is suitable for isolation and MPN enumeration. Selective-differential agars have also been found to enrich clinical over environmental strains. Warner and Oliver (200) determined through genotyping of the virulence correlating gene (*vcgC*) that CPC and mCPC agars preferentially select for clinical strains. Based upon the isolate enrichment results (Fig. 4-1), we can conclude that ratios of clinical to environmental strains in the seawater enrichments were reflective of the actual starting concentrations when using the APW10 medium.

For seawater, the lack of significant differences between enrichments having different salinities observed in samples collected in March (Fig. 7) might be explained by seasonal strain predominance differences (i.e. clinical versus environmental). Previous studies of Galveston Bay (53) have found that ‘environmental’ strains (i.e. 16S rRNA type A) predominate (up to 100%) in seawater and oysters from the early spring (March) to early summer (June) as water temperatures rise from below 10°C up to ~25°C and salinities are low (<10 PSU). Concentrations of *V. vulnificus* are also low in March (non-detectable to <10) and increase gradually with increasing water temperatures. Between late June and October, as temperatures increase to >25°C and salinities increase up to 30 PSU, there is a shift to a predominance (>70%) of ‘clinical’ strains (i.e. 16S rRNA type B, *vgcC* positive). Concentrations of *V. vulnificus* are also

highest in oysters (10^3 - 10^4 CFU/g) and seawater ($\sim 10^2$ CFU/ml) during this time. Then as temperatures begin to decrease again in early November ($<20^\circ\text{C}$) but salinities are still >20 PSU, the ratio of clinical to environmental strains is roughly even. In November, concentrations of *V. vulnificus* also decline rapidly with decreasing temperatures. The low concentration of *V. vulnificus* and the low percentage of clinical strains (i.e. %*vcgC*) in the March enrichments agree with the findings of these studies and we detected $<20\%$ *vcgC* genes for all enrichment types (Fig. 8) indicating a predominance ($>80\%$) of environmental strains.

Salinity appeared to be the key factor that influenced the concentration of *V. vulnificus* in the enrichments from seawater samples collected in September and November. These samples when enriched with the higher salinity broths (APW10, BHIB10, and LB10) had higher *vcgC* gene percentages than the broths having 5 PSU. The question is whether salinity is selecting for clinical strains or if clinical strains were already predominant in the original sample. The answer may be both. Ambient salinities in the seawater used for these enrichments were 10 to 18 PSU higher than in the March samples (~ 10 PSU). Again, Lin and Schwarz (53), who examined 16S rRNA types (A/B) in Galveston Bay seawater and oysters, found a predominance of 16S rRNA type B strains in August to November with salinities ranging from 21 to 29 PSU and temperatures ranging from 23°C to 30°C . Similar observations were also made when examining clinical versus environmental strains in Galveston Bay oysters. The 5 PSU salinity in the enrichment broths APW5 and BHIB5 may have selected for environmental strains (i.e. 16S rRNA type A), or may have limited growth of clinical

strains as percent *vcgC* genes was lower in these treatments. Chase and Harwood (185) who examined the influence of temperature and salinity on clinical strains of *V. vulnificus* found that highest growth rate (3.97 generations/hr) was achieved at temperature of 37°C and salinity of 25 PSU. Growth rate at the same temperature but at a lower salinity of 5 PSU was significantly lower (2.03 generations/hr). At 10 PSU, growth rate (3.24 generations/hr) was still significantly higher than at 5 PSU.

Enrichment with BHIB10 selected for a higher percentage of virulent clinical strains (i.e. *vcgC* positive) than the other enrichment media in the September samples. Percent clinical strains in the BHIB10 medium was ~20% higher than in the APW10 medium and ~30% higher than in the LB10 medium. All of these media contain 1% oligopeptides derived from digests of protein, but BHIB also contains 50% infusions of calf brains and beef hearts that increase the iron content by 17.5 µM. The high iron content in BHIB stimulates cytolysin/hemolysin activity of *V. vulnificus* (196, 206) and induces production of flagellar proteins that are integral for adhesion and biofilm formation in *V. vulnificus* (207). This trend was weakly continued in the November samples with only one sample (GB6; Fig. 8) having significantly higher percent clinical strains in the BHIB10 medium. It is apparent that salinity also plays a role in selecting for clinical strains since the BHIB5 medium with lower salinity had 50-60% lower percent clinical strains than BHIB10.

Different enrichment media selected for different ratios of clinical to environmental strains. However the enrichment of seawater results indicated that not only does the starting ratio matter, so does the salinity of the seawater inoculum. In other

words, seasonal differences in predominance of clinical or environmental strains in seawater influence the enrichment process. The selective preference of the BHIB10 medium for clinical strains indicates that iron plays an important role in the infection of the human host.

CHAPTER V

THE EFFECTS OF TEMPERATURE, PH AND IRON ON BIOFILM PRODUCTION BY *VIBRIO VULNIFICUS*

Overview

Due to the nature of *Vibrio vulnificus* infections, no studies of a biofilm-associated form in this pathogen's life cycle have been conducted. Biofilm formation by clinical and environmental reference strains of *V. vulnificus* were tested under different temperature (24°C, 30°C and 37°C), pH (5.5, 7.5, and 8.5) and iron concentration (18, 30, 50, 100, and 200 µM) conditions. For all strains, biofilm production at 24°C was often two to three times greater than at 30°C and 37°C, in multiple trials. The lowest amount of biofilm production was observed at 37°C ($p<0.001$). Biofilm production by clinical strains was consistently higher ($p<0.001$) at 24°C than environmental strains. Highest growth rates of all strains occurred at 37°C. The general trend for the pH trials was for highest biofilm production at pH 5.5 by all strains when incubated at 24°C. When incubated at 37°C, highest biofilm production at pH 5.5 was observed in primarily clinical isolates ($p<0.05$) indicating a greater resistance to the acidic environment of the host gastrointestinal tract. Growth rates were lowest at pH 5.5 for environmental strains but for clinical strains there were no differences at pH 5.5, 7.5, and 8.5 demonstrating a tolerance to acidic and alkaline conditions. There was a strong, direct correlation between iron concentration in the growth medium and biofilm production by all strains tested (clinical $R^2=0.91, 0.94, \text{ and } 0.98$; environmental $R^2=0.83, 0.84, 0.96$; $p<0.001$).

However, no differences were observed between clinical and environmental strains. With regards to temperature and pH, higher biofilm production appears to be a trait of clinical strains and could be considered a virulence factor. The results of this study indicate that biofilm production by *V. vulnificus* may be important to survival in the marine environmental and the human host.

Introduction

Vibrio vulnificus is a highly lethal, opportunistic human pathogen that is ubiquitous in marine coastal waters. Infection occurs from ingestion of raw or undercooked seafood or exposure of wounds to seawater containing this microorganism. Among healthy people *V. vulnificus* can cause severe gastrointestinal illness. In individuals with elevated serum iron or are immune-compromised, primary septicemia is the most lethal infection with a mortality rate of >50% (208, 209). Wound infections can progress rapidly to cellulites, bullae, and necrotizing fasciitis however the mortality rate is only about 25% (208). The mechanics of infection have not been well studied and it is not known if biofilm plays a role in the colonization of the human host or survival in the marine environment.

Vibrio cholerae exhibits biofilm mode of growth not only in the aquatic environment but also in the intestinal tract of its human host (49, 51). Unlike *V. cholerae*, *V. vulnificus* do not prefer living in the small intestine but instead colonize wounds and blood serum, therefore biofilm formation in *V. vulnificus* has received little attention in the clinical setting due to the nature of the infections. And, there are no data

for biofilm formation by *V. vulnificus* in the marine environment. Instead, studies of biofilm formation by *V. vulnificus* have focused on the identification of proteins essential for biofilm development (i.e. pili, flagella, and outer membrane) rather than on environmental factors contributing to biofilm development (31, 46, 47, 134).

Biofilm formation is considered a selective survival strategy of microbial communities for protection from nutrient deprivation, pH changes, oxygen radicals, disinfectants, and antibiotics (210), as well as for facilitating the complex dynamic interactions of close proximity between microorganisms, in particular the communication of the individual cells via signaling pathways (136). According to the CDC reports, approximately 65% of bacterial pathogens form biofilms and 80% according to National Institutes of Health (NIH). Biofilms are associated with a great number of serious chronic infections (141). Pathogens in biofilm exhibit more resistance to the clearance by human host defenses and antimicrobial treatments, since they are protected by the presence of the biofilm matrix that acts as a diffusion barrier (211, 212). Planktonic cells can be 1,000 times more susceptible to antibiotic treatments than biofilm associated cells (212, 213). The development of anti-biofilm therapeutics against biofilms is one of the most challenging tasks in medicine today (141).

There is a considerable body of data demonstrating that the biofilm formation depends on various physical, chemical and biological parameters such as nutrient availability, pH, salinity, temperature, iron concentration, oxygen, quorum sensing and genetics (157, 160, 161, 214-217). Hostacka et al. (160) compared the effects of temperature (30°C and 37°C) and pH (from 5.5 to 8.5) on biofilm formation in different

bacterial species *P. aeruginosa*, *Klebsiella pneumoniae*, and *Vibrio cholerae* non-O1 and O1, and observed that an increase in pH significantly affected the production of biofilm in all the bacterial species and strains tested. However temperature had no effect suggesting that genetic background plays a more important role in the development of biofilm than do environmental parameters. Di Bonaventura et al. (161) examined the effects of temperature, pH, and static-dynamic conditions on biofilm development of 51 *Stenotrophomonas maltophilia* clinical isolates. They observed higher biofilm formation at 32°C than at either 18°C or at 37°C, and the amount of biofilm produced was significantly higher at pH 7.5 and pH 8.5 than at pH 5.5. This study also indicated that strain-to-strain differentiations, regardless of environmental factors, have a marked effect on the biofilm development of *S. maltophilia*. While most clinical strains of *S. maltophilia* produced biofilms, 2% of the clinical strains did not form biofilm under the same conditions. Seemingly, not all clinical strains have the potential to form biofilm, and environmental parameters and host factors are also important to enable the individual strains to form biofilm.

Iron is one of the most important essential factors for the growth of microorganisms and involved in pathogenicity (208). Iron also plays an important role in biofilm development in several pathogens (157, 170, 171). Yang et al. (157) conducted a biofilm experiment to examine the effect of iron on biofilm development of *P. aeruginosa* and observed that 5 µM ferric chloride containing-media increased the biofilm mass of *P. aeruginosa* and the resistance to antimicrobials, while an increased concentration of iron up to 100 µM ferric chloride repressed the biofilm development

and exhibited decreased resistance to antimicrobial compounds. Musk et al. (170) also observed that an increase in iron concentration inhibited the expression of the genes required for biofilm formation of *P. aeruginosa* and addition of excess iron disrupted even the pre-existing biofilms. That study determined that a limited range of iron concentration from 1 μM to 100 μM is required for the development of *P. aeruginosa* biofilm. Data from another study by Martinez (214) revealed that although supplementation of culture medium with 100 μM iron dramatically increased the growth of individual *S. maltophilia* at 30°C and 35°C, elevated iron concentration in culture medium showed no decreasing or increasing effect on biofilm formation of *S. maltophilia*. On the other hand, *Staphylococcus aureus*, produces more biofilm with increasing iron concentration, up to 200 μM (165).

In this study, we examined the effect of temperature, pH, and iron concentration on *in vitro* biofilm production by *V. vulnificus*. We compared biofilm production by clinical strains, isolated from infected patients, to environmental strains, isolated oysters. Clinical strains are generally more virulent than environmental strains (23). Biofilm production is not a virulence factor, however we hypothesized that clinical strains would produce greater amounts of biofilm than environmental strains as a superior means of survival in the human host and the marine environment.

Materials and methods

Bacterial strains and growth conditions. Three clinical and three environmental reference strains (Table 4), obtained from Department of Health & Human Services (Dauphin Island, AL), were tested for biofilm production at different temperatures, pH and iron concentrations. Strains were maintained in Brain Heart Infusion Broth (BHIB; Bacto) at 37°C with shaking at 100 rpm with re-inoculation into fresh media every three days. In preparation for experimental trials, 100 µl from maintained cultures was inoculated into fresh BHIB (5 ml) and incubated for 12 hr at 37°C with shaking at 100 rpm. To ensure that no contamination of strain cultures had occurred, strains were streaked onto BHIB agar plates and single colonies were verified with PCR and vibrio specific PCR primers (84).

Table 4: *V. vulnificus* strains used in this study

Strain ID	Source	16S type
99623 DP-F5	environmental	AB
99581 DP-C7	environmental	A
98640 DP-E9	environmental	A
ORL 8074	clinical	B
BUF 7211	clinical	B
FLA 8869	clinical	B

Experimental design. Brain heart infusion broth (BHIB) + seawater (16 PSU, pH 7) was autoclaved (15 min) in borosilicate tubes with lids and allowed to stand at room temperature overnight to ensure sterility. For the pH experiments, BHIB was adjusted to 16 PSU with NaCl because adjustment of BHIB + seawater to pH 8.5

resulted in a precipitate. pH of BHIB + NaCl was adjusted to 5.5, 7.5 and 8.5 by adding HCl or NaOH and measured with a Thermo Scientific Orion 350 PerpHecT® LogR® Meter. After pH adjustment, the media was reautoclaved for 15 mins. After autoclaving, pH of the media (20 µl) was retested with pH paper to ensure that pH adjustments were maintained. For the iron experiments, iron concentration of the BHIB + seawater medium was measured with an ICPMS (Xseries 2, Thermo Scientific) equipped with a Xt cone and PC³ spray chamber kit. Iron was quantified according to a calibration curve produced with ferric chloride standards. The iron concentration of BHIB + seawater was determined to be 18 µM which was supplemented with filter sterilized (0.2 µm polyether sulfone membrane filter) ferric chloride to achieve the experimental concentrations.

To determine concentration of strains for experiments, 100 µl from log phase cultures was transferred to 96 well plates and the optical density (OD) was measured at 600 nm with a Synergy 2 Multi-Mode Microplate Reader (BioTek). Adjustments were made to the concentrations to achieve an OD_{600nm} of 0.6 ($\sim 1 \times 10^7$ cells/ml) for the starting inoculum. Starting inoculum (20 µl) was pipetted into the glass tubes containing 500 µl medium and the tubes were vortexed, then incubated for 48 hr under different temperature (24, 30, and 37°C), pH (5.5, 7.5, and 8.5), and iron concentration (18, 30, 50, 100, and 200 µM) conditions. Normal iron concentrations of human serum are 14-32 µM in males and 10-28 µM in females and in persons with hemochromatosis 40 to 50 µM (203, 204), so we performed our experiment at concentrations relevant to human serum. Higher concentrations were tested to determine possible negative effects. Each

experiment was performed in two to three independent trials with 6 replicates per treatment. Non-inoculated media served as controls.

Biofilm assay. The production of biofilm by different strains of *V. vulnificus* was determined using a modified crystal violet staining method (218). After the 48 hr incubation under the desired conditions, the culture medium in the borosilicate glass tubes was gently poured out not to disturb the biofilm ring. Each tube was gently washed four times with 2% NaCl to remove any non-adhered cells. Crystal violet (0.1%; 600 µl) was added to tubes and incubated 40 minutes at room temperature. Afterwards, the unbound crystal violet was carefully decanted and tubes were rinsed thoroughly 3-4 times with 2% NaCl to remove the unbound dye (Fig 9). Biofilm-bound crystal violet was eluted with 600 µl of 98% ethanol for 10 minutes. Aliquots (100 µl) of the eluted dye were transferred to 96 well plates and absorbance was measured at 570 nm using a Synergy 2 Multi-Mode Microplate Reader (Biotek). When necessary, the eluted dye was diluted with ethanol for measurements and the final absorbance value was multiplied by the dilution factor after subtracting the OD reading from the control tubes.

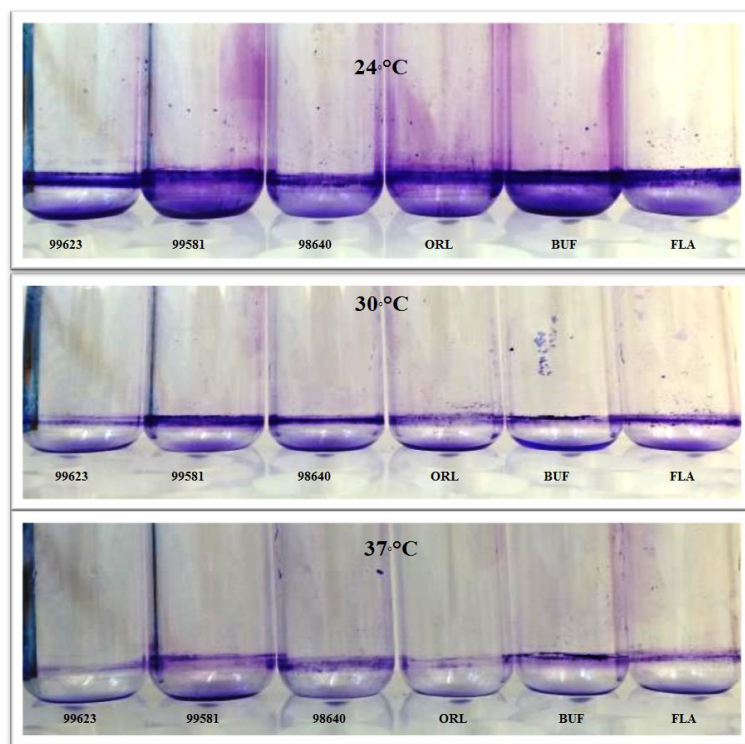


Figure 9. Biofilm stained with 0.1% crystal violet. The image shows stained aggregate rings in borosilicate glass culture tubes before addition of 600 μ l 98% EtOH. The greater thickness and darker color of the aggregate rings indicates higher biofilm formation produced by three environmental (numbers) and three clinical (letters) strains of *V. vulnificus* at 24°C, 30°C and 37°C, respectively.

Growth rates. Growth rates at different temperatures and pH were measured. All conditions were the same as in biofilm experiments except the starting amount of medium was 1000 μ l inoculated with 40 μ l inoculum and incubated for 12 hours. Aliquots (100 μ l) from tubes were transferred to 96 well plates every 4 hour and the OD was measured at 600 nm with a Synergy 2 Multi- Mode Microplate Reader (Biotek). Growth rate experiments were performed twice and the results were averaged. To convert OD to cell counts, *V. vulnificus* in aliquots were stained with DAPI (4',6-diamidino-2-phenylindole), filtered onto 0.2 μ m polycarbonate membranes, and then

counted with epifluorescence microscopy using a Zeiss Axioplan 2 microscope (Zeiss).

Growth rates were calculated with the following formula:

$$g = (\log_{10} N_t - \log_{10} N_0) / \log_{10}^2$$

N_t is the final cell count and N_0 is the starting cell count.

Statistical analysis. STATA (StataCorp) was used to evaluate statistical significance in the data. Bartlett's test was conducted *a priori* to one-way ANOVA to ensure equal variances and the Bonferroni test was conducted *post hoc* to determine differences in means; $p < 0.05$. Linear regression and the two-sample T-test were also used to evaluate the significance of the data; $p < 0.05$

Results

Effect of temperature. Three trials were conducted to test the effect of temperature (24°C, 30°C, and 37°C) on biofilm production by three clinical and three environmental strains of *V. vulnificus*. pH of media was 7. The results of trial 1 are presented in Fig. 10 and Table 5, trial 2 in Fig. 11 and Table 6, and trial 3 in Fig. 12, Table 7. In all three trials, the amount of biofilm produced by all strains were found to be significantly different at all temperatures tested (one-way ANOVA, $p < 0.001$). The amount of biofilm produced at 24°C by all strains was significantly higher ($p < 0.001$) than those formed at both 30°C and 37°C. The lowest amount of biofilm production was observed at 37°C ($p < 0.001$).

Comparison of combined clinical versus combined environmental strains (two-sample T-test, $p < 0.05$) found that mean differences of biofilm production by all clinical strains was significantly higher at 24°C than environmental strains in all three trials ($p < 0.001$) (Tables 8-10). However at higher temperatures the results were not consistent. At 30°C, clinical strains in trials 2 and 3 produced significantly greater amounts of biofilm than environmental strains ($p = 0.008$, $p = 0.032$, respectively). At 37°C, clinical strains produced higher biofilm than environmental strains only in trial 2 ($p = 0.002$).

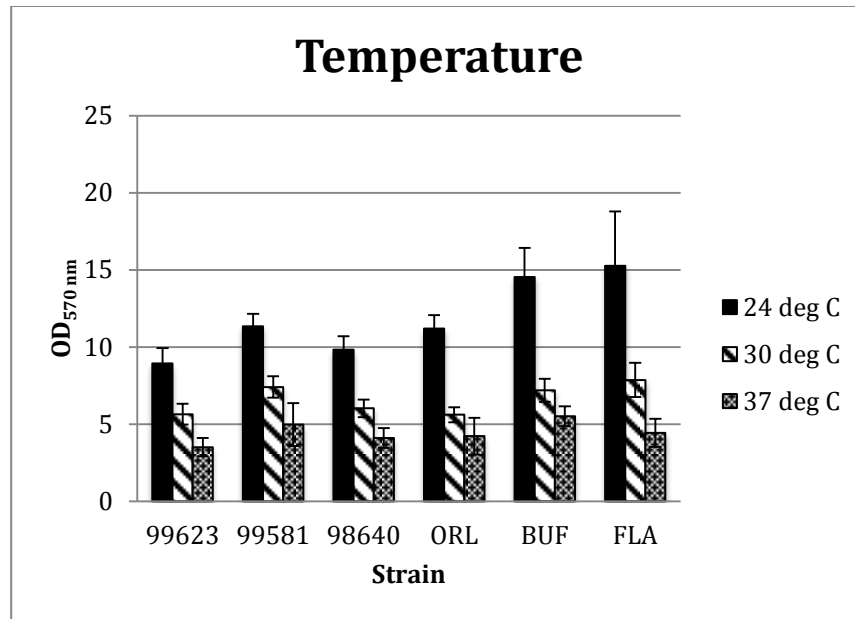


Figure 10. Effect of temperature (°C) on biofilm production (means \pm SD) by environmental (numbers) and clinical (letters) strains (1st trial).

Table 5. Effect of temperature on biofilm production by environmental (numbers) and clinical strains (letters); one-way ANOVA, means \pm SD; $p < 0.05$ (1st trial).

Strains	24°C	30°C	37°C	<i>p</i>
99623	8.9 \pm 1.0 ¹ *	5.6 \pm 0.7*	3.5 \pm 0.6*	<0.001
99581	11.3 \pm 0.9*	7.4 \pm 0.7*	4.9 \pm 1.4*	<0.001
98640	9.8 \pm 0.9*	6.0 \pm 0.6*	4.1 \pm 0.6*	<0.001
ORL	11.2 \pm 0.9*	5.6 \pm 0.5*	4.2 \pm 1.2*	<0.001
BUF	14.5 \pm 1.9*	7.2 \pm 0.7*	5.5 \pm 0.6*	<0.001
FLA	15.3 \pm 3.5*	7.9 \pm 1.1*	4.4 \pm 0.9*	<0.001

¹Optical density at 570 nm

*Significantly different as determined by Bonferroni *post hoc* test.

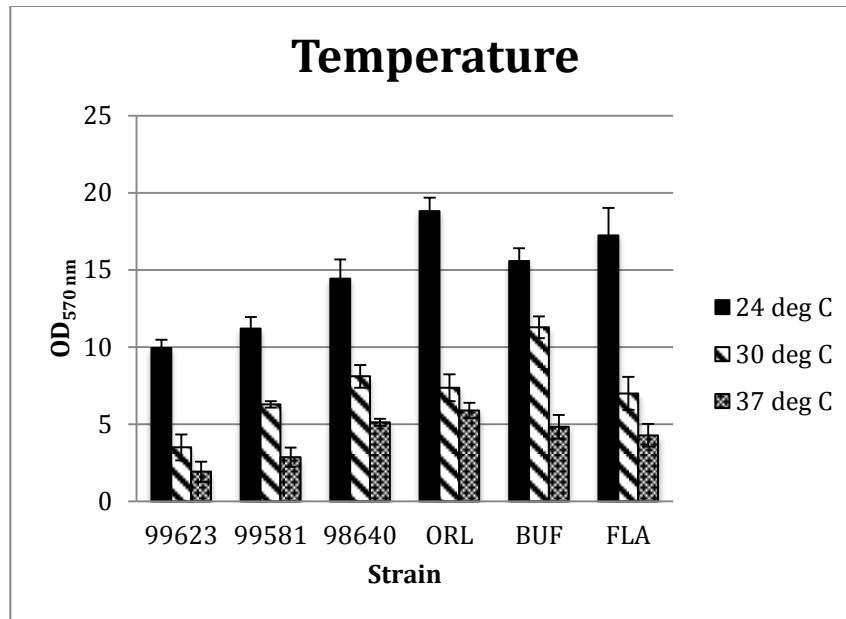


Figure 11. Effect of temperature (°C) on biofilm production (means \pm SD) by environmental (numbers) and clinical (letters) strains (2nd trial).

Table 6. Effect of temperature on biofilm production by environmental (numbers) and clinical strains (letters); one-way ANOVA, means \pm SD; $p < 0.05$ (2nd trial).

Strains	24°C	30°C	37°C	<i>p</i>
99623	9.9 \pm 0.5 ¹ *	3.5 \pm 0.8*	1.9 \pm 0.7*	<0.001
99581	11.2 \pm 0.8*	6.3 \pm 0.2*	2.9 \pm 0.6*	<0.001
98640	14.4 \pm 1.3*	8.1 \pm 0.7*	5.1 \pm 0.2*	<0.001
ORL	18.8 \pm 0.9*	7.4 \pm 0.9*	5.9 \pm 0.5*	<0.001
BUF	15.6 \pm 0.8*	11.3 \pm 0.7*	4.8 \pm 0.8*	<0.001
FLA	17.2 \pm 1.8*	6.9 \pm 1.1*	4.3 \pm 0.7*	<0.001

¹Optical density at 570 nm

*Significantly different as determined by Bonferroni *post hoc* test.

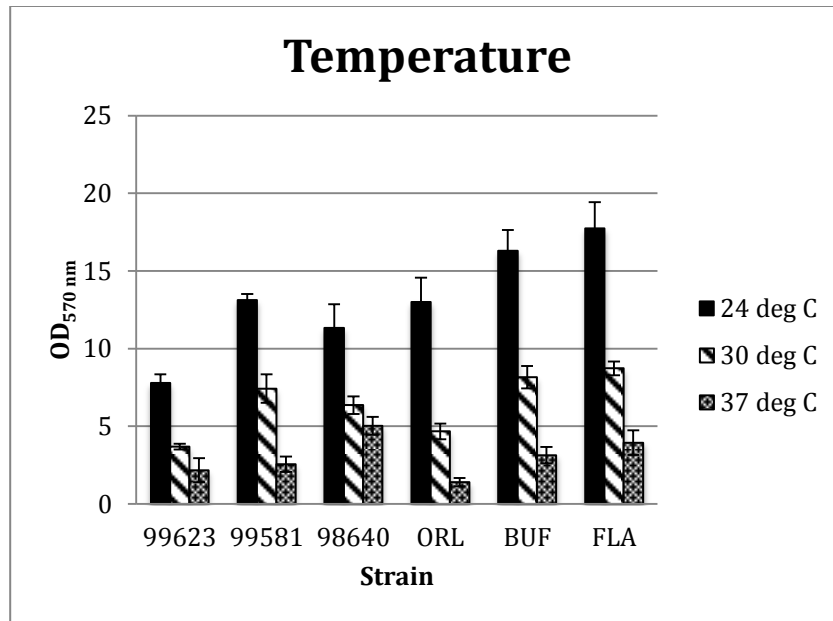


Figure 12. Effect of temperature (°C) on biofilm production (means \pm SD) by environmental (numbers) and clinical (letters) strain (3rd trial).

Table 7. Effect of temperature on biofilm production by environmental (numbers) and clinical strains (letters); one-way ANOVA, means \pm SD; $p < 0.05$ (3rd trial).

Strains	24°C	30°C	37°C	<i>p</i>
99623	7.8 \pm 0.6 ¹ *	3.7 \pm 0.2*	2.1 \pm 0.8*	<0.001
99581	13.1 \pm 0.4*	7.4 \pm 0.9*	2.6 \pm 0.5*	<0.001
98640	11.3 \pm 1.5*	6.4 \pm 0.6*	5.0 \pm 0.6*	<0.001
ORL	12.9 \pm 1.6*	4.7 \pm 0.5*	1.4 \pm 0.3*	<0.001
BUF	16.3 \pm 1.3*	8.2 \pm 0.7*	3.1 \pm 0.5*	<0.001
FLA	17.7 \pm 1.7*	8.7 \pm 0.4*	3.9 \pm 0.8*	<0.001

¹Optical density at 570 nm

*Significantly different as determined by Bonferroni *post hoc* test.

Table 8. Comparison of biofilm production by clinical vs environmental strains at different temperatures; two-sample T-test; means \pm SD; $p < 0.05$ (1st trial).

Temperature	Clinical	Environmental	<i>p</i>
24°C	13.6 \pm 2.9 ^{1*}	10.0 \pm 1.3	<0.001
30°C	6.9 \pm 1.2	6.4 \pm 0.9	0.166
37°C	4.7 \pm 1.1	4.2 \pm 1.1	0.149

¹Optical density at 570 nm

*Significantly different

Table 9. Comparison of biofilm production by clinical vs environmental strains at different temperatures; two-sample T-test; means \pm SD; $p < 0.05$ (2nd trial).

Temperature	Clinical	Environmental	<i>p</i>
24°C	17.2 \pm 1.8 ^{1*}	11.8 \pm 2.1	<0.001
30°C	8.5 \pm 2.2*	5.9 \pm 2.0	0.008
37°C	4.9 \pm 0.9*	3.3 \pm 1.5	0.002

¹Optical density at 570 nm

*Significantly different

Table 10. Comparison of biofilm production by clinical vs environmental strains at different temperatures; two-sample T-test; means \pm SD; $p < 0.05$ (3rd trial).

Temperature	Clinical	Environmental	<i>p</i>
24°C	15.7 \pm 2.5 ^{1*}	10.8 \pm 2.5	<0.001
30°C	7.2 \pm 1.9*	5.8 \pm 1.7	0.032
37°C	2.8 \pm 1.2	3.3 \pm 1.4	0.346

¹Optical density at 570 nm

*Significantly different

Effect of pH. The effect of pH on biofilms produced by environmental and clinical strains of *V. vulnificus* was tested in medium with pH 5.5, 7.5 and 8.5, incubated at 24°C, for 48 hr (Figs. 13-15, Tables 11-13). In trial 1, environmental strains 99623 and 99581 had significantly different biofilm production at all three pH ($p<0.001$) with highest production at pH 5.5. For environmental strain 98640, highest biofilm production was at pH 5.5. Clinical strain ORL also had significantly different biofilm production at all three pH values ($p<0.001$) with highest biofilm production at pH 5.5. And highest biofilm production for clinical strains BUF and FLA was highest at pH 5.5 and 8.5 ($p<0.001$). In trial 2, environmental strains 99623 and 99581 again had significantly different biofilm production at all three pH ($p<0.001$) with highest production at pH 5.5. However environmental strain 98640 had highest biofilm production at both pH 5.5 and 7.5. For clinical strain ORL, highest biofilm production was also at pH 7.5 and 5.5. Clinical strains BUF and FLA had highest biofilm production at pH 5.5 ($p<0.001$). In trial 3, all strains had highest biofilm production at pH 5.5. The overall trend from all trials was for highest biofilm production at pH 5.5. Comparison of clinical to environmental isolates (two-sample T-test) revealed that significantly higher biofilm production occurred with clinical isolates at all pH values, except in one case (pH 5.5) from trial 2 (Tables 14-16).

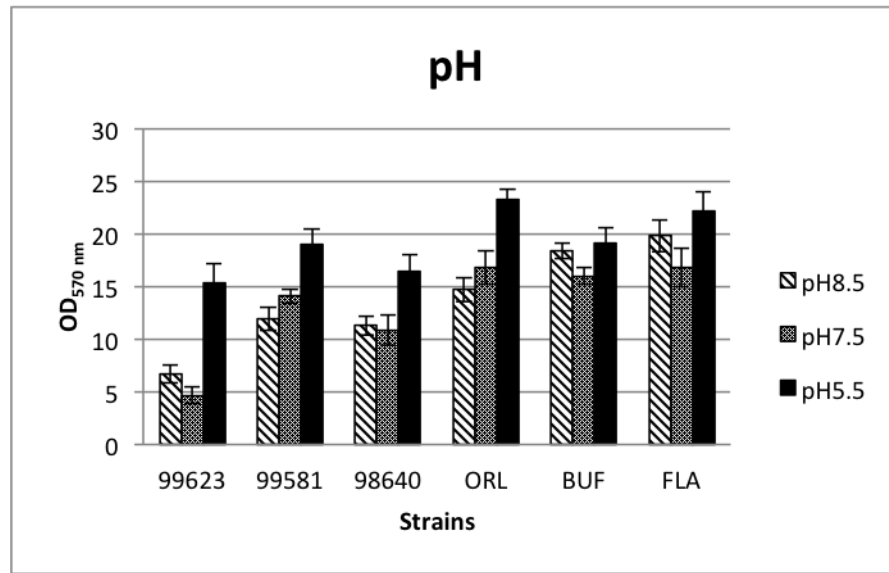


Figure 13. Biofilm production by environmental (numbers) and clinical (letters) strains of *V. vulnificus* at different pH; means \pm SD (1st trial).

Table 11. Effect of pH on biofilm production by three environmental (numbers) and three clinical (letters) strains; means \pm SD; one-way ANOVA; $p < 0.05$ (1st trial).

Strains	pH 5.5	pH 7.5	pH 8.5	<i>p</i>
99623	15.4 \pm 1.8 ^{1*}	4.7 \pm 0.8*	6.7 \pm 0.9*	<0.001
99581	18.9 \pm 1.6*	14.1 \pm 0.7*	11.9 \pm 1.1*	<0.001
98640	16.5 \pm 1.5*	10.9 \pm 1.4	11.3 \pm 0.9	<0.001
ORL	23.3 \pm 0.9*	16.8 \pm 1.6*	14.7 \pm 1.1*	<0.001
BUF	19.2 \pm 1.4	15.9 \pm 0.8*	18.4 \pm 0.8	<0.001
FLA	22.2 \pm 1.7	16.8 \pm 1.8*	19.8 \pm 1.5	<0.001

¹Optical density at 570 nm

*Significantly different as determined by Bonferroni *post hoc* test.

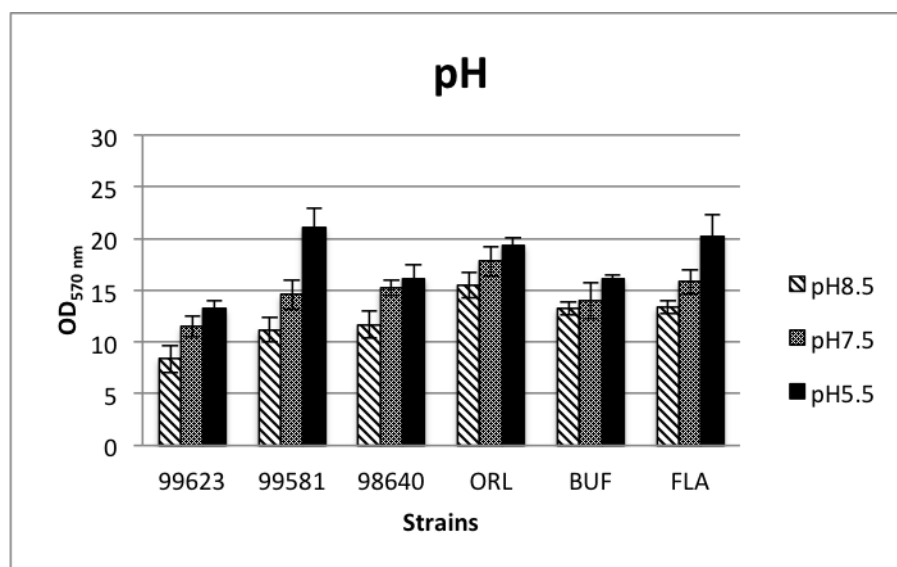


Figure 14. Biofilm production by environmental (numbers) and clinical (letters) strains of *V. vulnificus* at different pH; means \pm SD (2nd trial).

Table 12. Effect of pH on biofilm production by three environmental (numbers) and three clinical (letters) strains; means \pm SD; one-way ANOVA; $p < 0.05$ (2nd trial).

Strains	pH 5.5	pH 7.5	pH 8.5	<i>p</i>
99623	13.2 \pm 0.8 ¹ *	11.5 \pm 0.9*	8.4 \pm 1.3*	<0.001
99581	21.0 \pm 1.9*	14.6 \pm 1.4*	11.2 \pm 1.2*	<0.001
98640	16.2 \pm 1.4	15.2 \pm 0.7	11.7 \pm 1.3*	<0.001
ORL	19.4 \pm 0.8	17.8 \pm 1.4	15.5 \pm 1.2*	<0.001
BUF	16.1 \pm 0.3*	13.9 \pm 1.7	13.2 \pm 0.6	<0.001
FLA	20.2 \pm 2.2*	15.8 \pm 1.2*	13.4 \pm 0.6*	<0.001

¹Optical density at 570 nm

*Significantly different as determined by Bonferroni *post hoc* test.

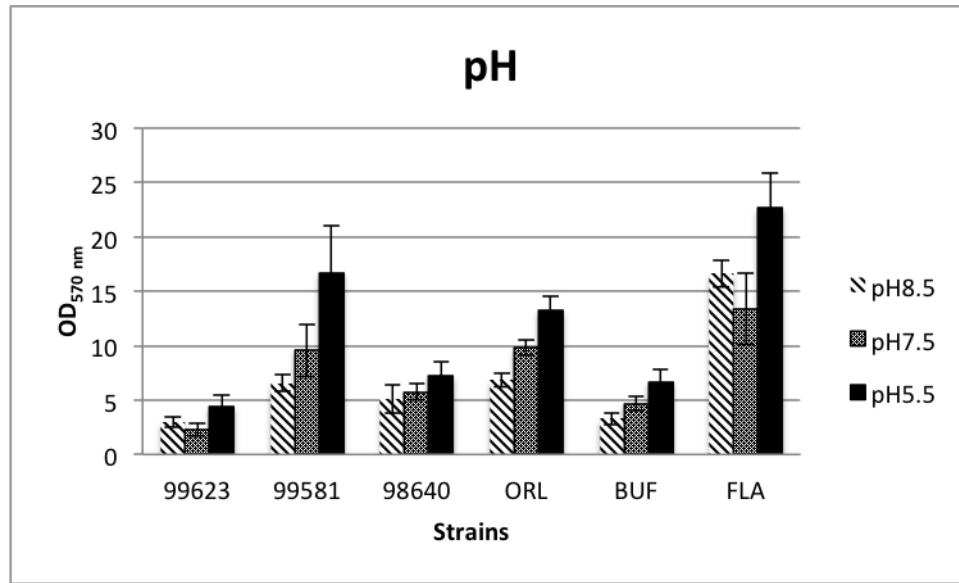


Figure 15. Biofilm production by environmental (numbers) and clinical (letters) strains of *V. vulnificus* at different pH; means \pm SD (3rd trial).

Table 13. Effect of pH on biofilm production by three environmental (numbers) and three clinical (letters); one-way ANOVA, means \pm SD; $p < 0.05$ (3rd trial).

Strains	pH 5.5	pH 7.5	pH 8.5	<i>p</i>
99623	4.4 \pm 1.0 ¹ *	2.3 \pm 0.6	2.9 \pm 0.5	<0.001
99581	16.7 \pm 4.4*	9.5 \pm 2.4	6.5 \pm 0.8	<0.001
98640	7.2 \pm 1.3*	5.7 \pm 0.7	5.1 \pm 1.3	<0.05
ORL	13.2 \pm 1.3*	9.8 \pm 0.7*	6.8 \pm 0.6*	<0.001
BUF	6.6 \pm 1.2*	4.7 \pm 0.7*	3.3 \pm 0.6*	<0.001
FLA	22.7 \pm 3.2*	13.4 \pm 3.3	16.6 \pm 1.2	<0.001

¹Optical density at 570 nm

*Significantly different as determined by Bonferroni *post hoc* test.

Table 14. Biofilm production by clinical vs environmental strains at different pH; two-sample T-test; means \pm SD, $p<0.05$ (1st trial).

pH	Clinical	Environmental	<i>p</i>
5.5	21.5 \pm 2.2 ¹ *	16.9 \pm 2.2	<0.001
7.5	16.6 \pm 1.5*	9.9 \pm 4.1	<0.001
8.5	17.7 \pm 2.5*	9.9 \pm 2.6	<0.001

¹Optical density at 570 nm

*Significantly different

Table 15. Biofilm production by clinical vs environmental strains at different pH; two-sample T-test; means \pm SD, $p<0.05$ (2nd trial).

pH	Clinical	Environmental	<i>p</i>
5.5	18.6 \pm 2.2 ¹	16.8 \pm 3.6	0.082
7.5	15.9 \pm 2.1*	13.7 \pm 1.9	<0.005
8.5	14.0 \pm 1.3*	10.4 \pm 1.9	<0.001

¹Optical density at 570 nm

*Significantly different

Table 16. Biofilm production by clinical vs environmental strains at different pH; two-sample T-test; means \pm SD, $p<0.05$ (3rd trial).

pH	Clinical	Environmental	<i>p</i>
5.5	14.1 \pm 7.1 ¹ *	9.4 \pm 5.9	<0.05
7.5	9.3 \pm 4.1*	5.9 \pm 3.3	<0.01
8.5	8.9 \pm 5.9*	4.9 \pm 1.7	<0.01

¹Optical density at 570 nm

*Significantly different

Effect of pH at 37°C. We tested biofilm production by clinical and environmental strains of *V. vulnificus* at different pH but incubated at 37°C, the average human body temperature. In trial 1 (Fig. 16, Table 17) no significant differences were observed between pH values for all environmental strains. Within clinical strains, isolate BUF produced a significantly greater amount of biofilm at pH 5.5 (6.0 ± 0.3 ; $p < 0.001$) while there were no differences for the other clinical strains ORL, and FLA. In trial 2 (Fig. 9, Table 15) within environmental strains, isolate 99581 produced significantly greater biofilm at pH 5.5 than the other isolates. Isolate 98640 produced more biofilm at pH 5.5 and 7.5 (4.3 ± 0.7 and 3.8 ± 0.3 , respectively; $p = 0.0002$). Within clinical strains, isolates BUF and FLA produced more biofilm at pH 5.5 (11.7 ± 0.7 and 4.5 ± 0.4 ; $p < 0.0010$) than at pH 7.5 and 8.5. A two-sample T-test comparison of clinical to environmental isolates (Tables 19 and 20) found that clinical isolates produced significantly more biofilm at pH 5.5 and 8.5 ($p = 0.016$) in trial 1. And, in trial 2, clinical strains produced more biofilm at pH 5.5 (6.3 ± 3.9 ; $p = 0.0488$) than environmental isolates.

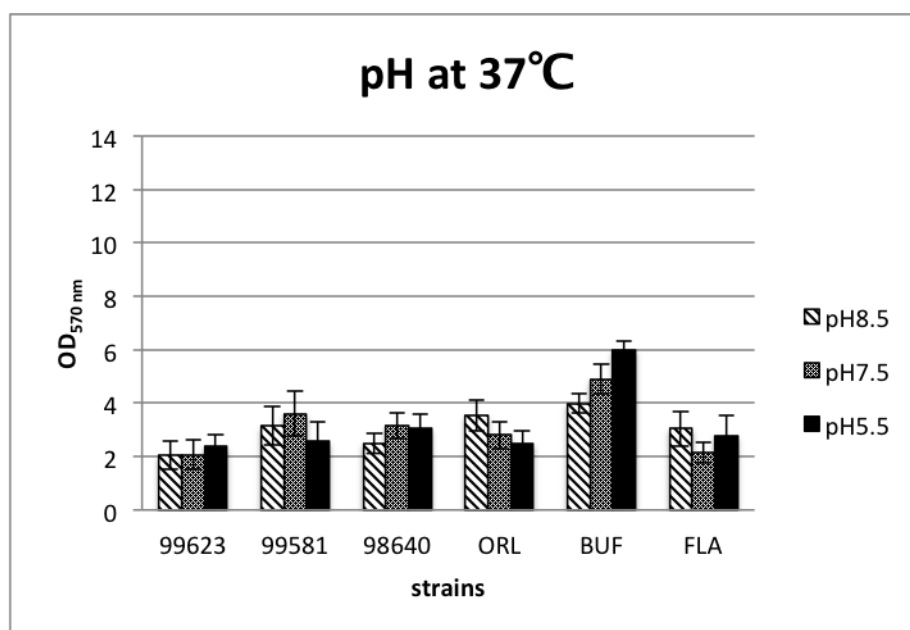


Figure 16. Biofilm production by environmental (numbers) and clinical (letters) strains of *V. vulnificus* at different pH incubated at 37°C; means±SD (1st trial).

Table 17. Effect of pH incubated at 37°C on biofilm production by three environmental (numbers) and three clinical (letters); one-way ANOVA, means ± SD; $p < 0.05$ (1st trial).

Strains	pH 5.5	pH 7.5	pH 8.5	<i>p</i>
99623	2.3±0.4 ¹	2.1±0.6	2.0±0.5	0.4825
99581	2.5±0.7	3.6±0.8	3.1±0.7	0.1028
98640	3.1±0.5	3.2±0.5	2.5±0.4	0.0609
ORL	2.5±0.5	2.8±0.5	3.5±0.6	0.0516
BUF	6.0±0.3*	4.9±0.5	3.9±0.4	<0.001
FLA	2.8±0.8	2.1±0.4	3.0±0.7	0.0666

¹Optical density at 570 nm

*Significantly different as determined by Bonferroni *post hoc* test.

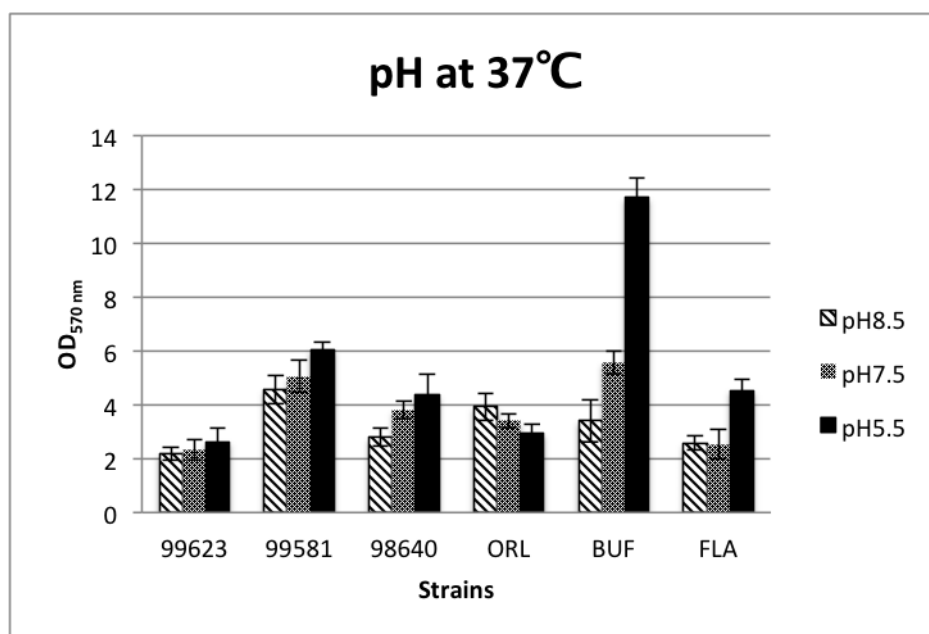


Figure 17. Biofilm production by environmental (numbers) and clinical (letters) strains of *V. vulnificus* at different pH incubated at 37°C; means±SD (2nd trial).

Table 18. Effect of pH incubated at 37°C on biofilm production by three environmental (numbers) and three clinical (letters); one-way ANOVA, means ± SD; $p < 0.05$ (2nd trial).

Strains	pH 5.5	pH 7.5	pH 8.5	<i>p</i>
99623	2.6±0.5 ¹	2.3±0.4	2.2±0.2	0.1454
99581	6.1±0.3*	5.1±0.6	4.6±0.5	0.0003
98640	4.3±0.7	3.8±0.3	2.8±0.3*	0.0002
ORL	2.9±0.3	3.4±0.2	3.9±0.5*	0.0018
BUF	11.7±0.7*	5.6±0.4*	3.4±0.8*	<0.001
FLA	4.5±0.4*	2.5±0.6	2.6±0.3	<0.001

¹Optical density at 570 nm

*Significantly different as determined by Bonferroni *post hoc* test.

Table 19. Biofilm production by clinical vs environmental strains at different pH incubated at 37°C; two-sample T-test; means±SD, $p<0.05$ (1st trial).

pH	Clinical	Environmental	<i>p</i>
5.5	3.8±1.7 ^{1*}	2.6±0.7	0.016
7.5	3.3±1.3	2.9±0.9	0.363
8.5	3.5±0.6*	2.7±0.6	0.0002

¹Optical density at 570 nm

*Significantly different

Table 20. Biofilm production by clinical vs environmental strains at different pH incubated at 37°C; two-sample T-test; means±SD, $p<0.05$ (2nd trial).

pH	Clinical	Environmental	<i>p</i>
5.5	6.3±3.9 ^{1*}	4.3±1.5	0.0488
7.5	3.8±1.3	3.7±1.2	0.7941
8.5	3.3±0.8	3.1±1.1	0.6727

¹Optical density at 570 nm

*Significantly different

Effect of iron. The effect of iron on biofilm production by *V. vulnificus* was tested by the addition of ferric chloride (FeCl₃) at 18, 30, 50, 100, and 200 µM. Linear regression analysis revealed that biofilm formation increased with increasing ferric chloride concentration (Figs. 18 and 19). The means of biofilm production with standard deviations are summarized in Tables 21 and 22. In trial 1, R² values of clinical strains ORL, BUF, FLA were high (0.98, 0.91, 0.94, respectively, $p<0.001$) indicating a strong correlation between biofilm production and iron concentration. R² value for environmental strain 98640 was also high (0.96) but was lower for environmental strains 99623 and 99581 (0.83 and 0.89, respectively). R² values in trial 2 were moderate for all strains ranging from 0.75 to 0.86. The overall trend from both trials indicated that

biofilm production increases with increasing iron concentration with no repression at higher concentrations (>50 μM). Comparison of clinical to environmental strains (two-sample T-test) found no differences in biofilm production as a response to increasing iron concentration (data not shown).

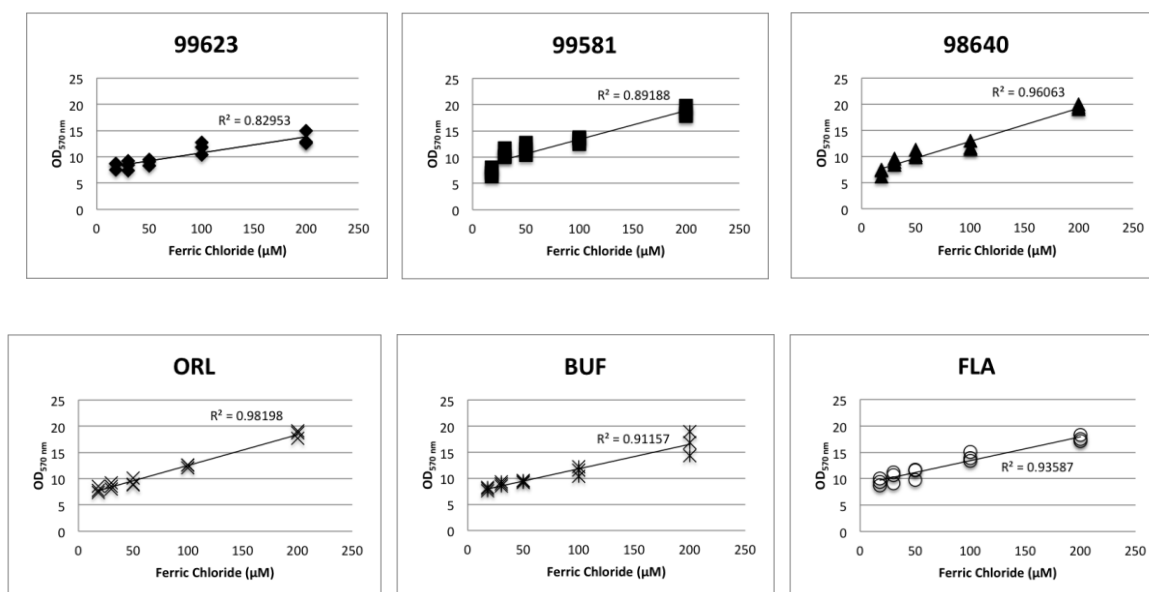


Figure 18. Biofilm production by environmental (numbers) and clinical (letters) strains of *V. vulnificus* with increasing ferric chloride concentrations according to regression analysis (1st trial).

Table 21. Effect of ferric chloride on biofilm production by environmental (numbers) and clinical (letters) strains; linear regression, means \pm SD, $p < 0.05$ (1st trial).

strains	18 μM	30 μM	50 μM	100 μM	200 μM	<i>p</i>	R^2
99623	8.3 \pm 0.7 ¹	8.3 \pm 0.9	8.9 \pm 0.6	11.6 \pm 1.2	13.5 \pm 1.3	<0.001	0.83
99581	7.2 \pm 0.9	10.7 \pm 0.9	11.6 \pm 1.2	13.3 \pm 0.7	18.7 \pm 0.9	<0.001	0.89
98640	6.9 \pm 0.7	8.9 \pm 0.6	10.4 \pm 0.7	12.0 \pm 0.9	19.5 \pm 0.5	<0.001	0.96
ORL	7.9 \pm 0.6	8.6 \pm 0.6	9.4 \pm 0.7	12.4 \pm 0.3	18.5 \pm 0.8	<0.001	0.98
BUF	7.9 \pm 0.3	8.9 \pm 0.3	9.4 \pm 0.2	11.4 \pm 0.9	16.7 \pm 2.3	<0.001	0.91
FLA	9.4 \pm 0.6	10.3 \pm 1.0	11.1 \pm 1.1	14.1 \pm 0.8	17.6 \pm 0.6	<0.001	0.94

¹Optical density at 570 nm

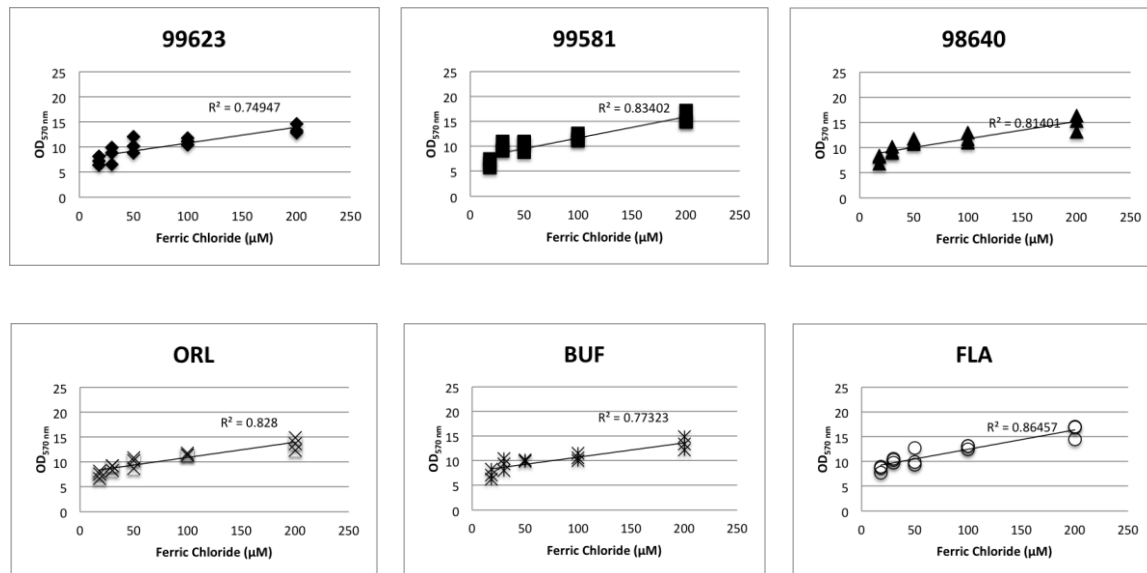


Figure 19. Biofilm production by environmental (numbers) and clinical (letters) strains of *V. vulnificus* with increasing ferric chloride concentrations (2nd trial).

Table 22. Effect of ferric chloride on biofilm production by environmental (numbers) and clinical (letters) strains; linear regression, means \pm SD, $p < 0.05$ (2nd trial).

Strains	18 μM	30 μM	50 μM	100 μM	200 μM	<i>p</i>	R^2
99623	7.2 \pm 0.8 ¹	8.4 \pm 1.7	10.4 \pm 1.6	11.1 \pm 0.7	13.6 \pm 0.9	<0.001	0.75
99581	6.6 \pm 0.9	9.9 \pm 1.	10.1 \pm 1.2	11.8 \pm 0.7	15.7 \pm 1.2	<0.001	0.83
98640	7.8 \pm 0.9	9.5 \pm 0.7	11.2 \pm 0.5	11.9 \pm 1.0	14.9 \pm 1.6	<0.001	0.81
ORL	7.5 \pm 0.7	8.9 \pm 0.5	9.9 \pm 1.1	11.5 \pm 0.3	13.6 \pm 1.3	<0.001	0.83
BUF	7.3 \pm 1.0	9.3 \pm 1.2	9.9 \pm 0.1	10.7 \pm 0.8	13.5 \pm 1.4	<0.001	0.77
FLA	8.5 \pm 0.7	10.2 \pm 0.4	10.7 \pm 1.8	12.9 \pm 0.4	16.1 \pm 1.5	<0.001	0.86

¹Optical density at 570 nm

Growth rate. Growth rates of environmental and clinical strains were determined during biofilm production at different temperatures and pH. Although the highest biofilm production occurred at 24°C, the highest growth rate was observed at 30 and 37°C for most strains (Table 23). For all environmental strains, lowest growth rate was observed at pH 5.5 with highest growth rates at pH 7.5 (Table 24). No significant differences were observed in the growth rates of the clinical strains at different pH values.

Table 23. Growth rates (generations/hr) of environmental (numbers) and clinical (letters) strains grown at different temperatures; one-way ANOVA, means \pm SD, $p < 0.05$.

Strain	24°C	30°C	37°C	<i>p</i>
99623	0.329 \pm 0.001*	0.390 \pm 0.005	0.425 \pm 0.012	0.0028
99581	0.339 \pm 0.016*	0.403 \pm 0.003	0.426 \pm 0.000	0.0059
98640	0.339 \pm 0.016	0.375 \pm 0.007	0.425 \pm 0.003*	0.0083
ORL	0.346 \pm 0.012*	0.397 \pm 0.006	0.423 \pm 0.003	0.0059
BUF	0.350 \pm 0.003*	0.415 \pm 0.005	0.432 \pm 0.008	0.0232
FLA	0.361 \pm 0.002*	0.412 \pm 0.001*	0.425 \pm 0.002*	0.0002

*Significantly different as determined by Bonferroni *post hoc* test.

Table 24. Growth rates (generations/hr) of environmental (numbers) and clinical (letters) strains grown at different pH; one-way ANOVA, means \pm SD, $p < 0.05$.

Strain	5.5	7.5	8.5	<i>p</i>
99623	0.306 \pm 0.001*	0.407 \pm 0.005*	0.358 \pm 0.016*	0.0048
99581	0.330 \pm 0.01*	0.436 \pm 0.002	0.400 \pm 0.015	0.0052
98640	0.350 \pm 0.017*	0.421 \pm 0.006	0.398 \pm 0.003	0.0170
ORL	0.358 \pm 0.026	0.431 \pm 0.014	0.392 \pm 0.004	0.0527
BUF	0.368 \pm 0.03	0.442 \pm 0.001	0.418 \pm 0.01	0.0587
FLA	0.375 \pm 0.04	0.437 \pm 0.036	0.408 \pm 0.018	0.3120

*Significantly different as determined by Bonferroni *post hoc* test.

Discussion

For all strains, biofilm production at 24°C was often two to three times greater than at 30 and 37°C in all three trials. Higher amount of biofilm production at lower temperature may be explained by high-frequency spontaneous phase variation of *V. vulnificus*. Grau et al. (218) defined a new phenotypic variant of this bacterium called the rugose variant or phenotype which is formed from opaque and translucent strains when they are continually cultivated at lower temperatures. This variant makes copious amounts of biofilms at the air-broth-glass interface as well as an aggregate ring. The highest biofilm production at 24°C in our study is consistent with these findings. However highest growth rate for most strains occurred at 30°C and 37°C, thus we assume that biofilm production is more related to switching events between different phenotypes of *V. vulnificus* rather than growth kinetics. Also, it is more likely that more cellular energy is devoted to biofilm production at 24°C than to cell replication. Upon comparing clinical and environmental strains, we observed that clinical strains produced significantly more biofilm than environmental strains at 24°C ($p < 0.001$). However at 30°C and 37°C biofilm production was not different, at least half the time. We propose that clinical strains of *V. vulnificus* form biofilms for protection in natural marine environments, when temperatures fall below 25°C. No data is available for *V. vulnificus* in this regard, but one study highlighted the importance of biofilm forming potential of *Vibrios* for their colonization and extended survival in the *ex vivo* marine environment (51). In experiments similar to ours, Townsley and Yildiz (159) tested biofilm formation by *V. cholerae* and found that biofilms produced at low temperatures (15°C and 25°C),

like those experienced in the aquatic habitat, had increased biomass and thickness and were more structured compared with those formed at 37°C. Another study (160) determined that *V. cholerae* produced slightly higher biofilm even at 30°C compared to 37°C. It is also the rugose variant of *V. cholerae* that produces well-developed biofilms and has increased levels of resistance to several aquatic environmental stresses (219). Although biofilm production by all strains was lower at 37°C, this result should not be overlooked since it demonstrates the possibility of a biofilm form for *V. vulnificus* in the human host that would be important for survival in the gastrointestinal tract. In *V. cholerae*, biofilm architecture is essential for survival in the stomach and then for attachment in the small intestine (50). And, in biofilm form, *V. cholerae* virulence is several orders of magnitude higher than in planktonic forms (49, 50).

V. vulnificus, like many gram negative bacteria, possesses several enzymes that neutralize low pH environments by breaking down amino acids to amines and CO₂ as well as scavenge for superoxide radicals (208). We found that all *V. vulnificus* strains produced about 33% more biofilm at pH 5.5 than at pH 7.5 or 8.5 (incubated at 24°C), which may be an additional protective response at lower pH. *V. cholerae* produces biofilm for protection in the presence of bile acids (162) that normally restrict bacterial growth in the small intestine (163). pH effects attachment of *V. cholerae* to surfaces. Attachment and adhesion to inert or living surfaces are the first steps for biofilm formation. Attachment rate of *V. cholerae* to zooplankton was examined under various pH conditions and the optimum attachment was observed at lower pH scale (pH 2) while increasing pH decreased the attachment rate (34). We did not examine attachment rate in

V. vulnificus but the production of biofilm in the gastrointestinal tract could facilitate attachment to the intestinal epithelium. Our work with *V. vulnificus* also found that more virulent clinical strains produced significantly more (25-50%) biofilm at all pH values tested when compared to less virulent environmental strains. In a similar study, a subset of more virulent strains, including a hyper-virulent ST-17 clone, of *Streptococcus agalactiae* were found to produce more biofilm under acidic pH than the rest of the strains tested (220). And, in our study, unlike environmental strains that had lower growth rates at pH 5.5, growth rates of clinical strains were not different at pH 5.5, 7.5 or 8.5 indicating a resistance to acidic and alkaline environments.

At 37°C, the average temperature of the human body, both clinical and environmental strains produced biofilm at all pH values (Tables 14 and 15). These findings are promising because the acidic gastrointestinal tract may drive clinical strains of *V. vulnificus* to form biofilm-like aggregates, as in the case in *V. cholerae* infections. One environmental isolate and two clinical isolates produced significantly higher biofilm at pH 5.5 with one clinical isolate (BUF) producing roughly two times more biofilm than all other strains. And, clinical strains, combined, produced more biofilm at pH 5.5 than environmental isolates indicating their greater resistance to a low pH environment such as the stomach and small intestine. These differences could be translated as increased virulence in these strains. There is evidence for higher virulence associated with increased biofilm production in *V. cholerae*. Wild type strains O395 and El Tor 6706, classical O1 and El Tor O1 biotypes, respectively, responsible for the last seven cholera pandemics, produce significantly more biofilm than less virulent variant strains (221).

Iron regulates biofilm formation by several human pathogens (157, 165-167). Our study found a direct correlation between free iron concentration in growth medium and biofilm production by *V. vulnificus*, although there were differences in the R^2 values between trials. We did not observe any negative effects of elevated iron concentrations (i.e. 50, 100 and 200 μM) on biofilm production, instead higher amounts of biofilm were produced confirming the ferrophilic nature of *V. vulnificus* (116) and provides a possible *in vivo* reaction to high serum iron concentrations. To our knowledge there are no data regarding biofilm formation by *V. vulnificus* in human serum. However, biofilms may play a role in attachment to the intestinal epithelium and subsequent necrosis. Biofilm may also be necessary for colonization of open or bleeding wounds. It is known that bacterial biofilms colonize wounds (222) and prevent the healing process by producing proteases thereby prolonging the inflammatory response (223). Bacterial cells in biofilm are also significantly more resistant to antibiotics and host immune defenses than planktonic cells and persist in wounds (134, 222, 224). *S. aureus*, that forms biofilms in chronic wounds (168) and is one of the most frequently isolated pathogens associated with nosocomial sepsis (169), also produces more biofilm with increasing iron concentration, up to 200 μM (165). However, other pathogens like *P. aeruginosa* have limits and produce begin to produce less biofilm at higher concentrations $>100 \mu\text{M}$ (157).

Non-transferrin bound iron is necessary for *V. vulnificus* growth in human serum. Kim et al. (116) determined that non-transferrin bound iron is required for initiation of *V. vulnificus* growth. *V. vulnificus* is killed by serum from healthy individuals but grows

rapidly in serum from patients with high concentrations of free iron (225, 226). In patients with hemochromatosis, the serum contains a pool of freely available iron (227). During infection, iron availability increases as intracellular iron is released by cell destruction by *V. vulnificus*-specific hemolysin/cytolysin. Iron stimulates transcription of the *vhA* gene that encodes for more hemolysin/cytolysin and is required for the efficient transcription of extracellular proteases that are also involved in cell destruction (196, 207).

Free iron is also important for the formation of biofilm by *V. cholerae* and is required for switching from smooth cells to the rugose variant that produces the EPS matrix of biofilm (167). The loss of the flagellum in the switching from smooth to rugose variant triggers the production of EPS by *V. cholerae* (228). And in non-motile, rugose *V. cholerae*, production of toxin-coregulated pili, cholera toxin, and cell-associated hemolysin are increased (229). Iron also controls the adhesion of *V. vulnificus* to biotic and abiotic surfaces. There is a direct correlation between iron content in growth medium and production of flagellar proteins that are integral for adhesion and biofilm formation in *V. vulnificus* (207). A comprehensive study is needed to explain the role of iron in development of biofilm by *V. vulnificus* with consideration of high-frequency phenotypic switching events from smooth or opaque phenotypes to the rugose variant.

Due to the nature of *V. vulnificus* infections, no studies of a biofilm form in this pathogen's life cycle have been conducted. The results of our study bring to light the possibility of biofilm formation by *V. vulnificus* in the marine environment as well as *in*

vivo. Although all strains produced biofilms at 37°C, host body temperature, higher biofilm was produced at 24°C, a temperature that would be encountered in the marine environment. We did not test substrate but did observe that no biofilm is formed by *V. vulnificus* on plastic surfaces. Substrate type may be key to observing biofilm formation by *V. vulnificus* in the marine environment. There was a trend for acidic pH, typical of the gastrointestinal tract, to stimulate highest biofilm production by both clinical and environmental strains but clinical strains produced more biofilm both at 24°C and 37°C. Free iron, typical of hemochromatosis, also plays a strong role in biofilm production in *V. vulnificus*, which may facilitate infection of open wounds and perhaps in blood. Higher biofilm production appears to be a trait of clinical strains and could be considered a virulence factor. The results of this study indicate that biofilm production by *V. vulnificus* may be important to survival in the marine environmental and the human host.

CHAPTER VI

DIFFERENTIAL EXPRESSION OF *VVHA* AND *CPS OPERON ALLELE 1* GENES IN *VIBRIO VULNIFICUS* UNDER BIOFILM AND PLANKTONIC CONDITIONS

Overview

Examination of genes encoding for the virulence factors, hemolysin/cytolysin (*vvhA*) and capsular polysaccharide (*CPS allele 1*), during biofilm formation revealed that their expression was influenced by maturity of the biofilm as well as by temperature. At 24°C, expression of *vvhA* during biofilm formation was low between 4 and 12 h but increased 10 fold by 24 h to $(5.1 \times 10^4 \pm 6.3 \times 10^3)$ mRNA copies/ml as the biofilm matured. Compared to planktonic cells, expression of *vvhA* during biofilm formation at 24°C was initially up-regulated at 4 h (1.07 ± 0.00 -fold) but then was down-regulated almost four-fold during the intermediate and mature stages of biofilm formation. In contrast, *vvhA* expression at 37°C was up-regulated almost four-fold in the early stages (4 and 6 h) of biofilm formation and remained two-fold up-regulated by 24 h even as the biofilm was deteriorating. *CPS allele 1* expression at 24°C during biofilm formation was up-regulated ($+1.50 \pm 0.18$ -fold) during the initial attachment phase of the cells but was strongly down-regulated during the intermediate phases at 8 and 10 h (74.42 ± 42.16 -fold and -453.76 ± 193.32 -fold, respectively) indicating that capsular polysaccharide (CPS) is not important to intermediate biofilm architecture. Interestingly, as the biofilm matured by 24 h, expression of *CPS allele 1* was again up-regulated ($+1.88 \pm 1.07$) indicating that

CPS plays a role in mature biofilm. At 37°C, CPS allele 1 expression was significantly up-regulated (up to 10⁵) during biofilm formation indicating that the biofilm form of *V. vulnificus* may be preferred over the planktonic form in the human host.

Introduction

Vibrio vulnificus a naturally occurring marine pathogen accounts for 95% of all seafood related deaths in the United States, and causes the highest-death rate of any food-borne disease (8, 21). Forms of illness after ingestion of *V. vulnificus* are typically gastrointestinal (gastroenteritis) presented as vomiting, diarrhea, and severe abdominal pain, but if not treated, can rapidly infect the bloodstream (septicemia) within 24 to 48 hr resulting in fever, chills, blistering skin lesions (bullae), tissue necrosis, and death (2, 13, 67). Infections can also be caused by exposure of pre-existing or fresh wounds to seawater containing *V. vulnificus* or by handling seafood (2). The mortality rate for primary septicemia, the most lethal form of infection, is greater than 50% after consumption of raw or undercooked oysters having high enough titers of *V. vulnificus* cells, and it can result in higher mortality rate in people who are immuno-compromised or have an underlying disease such as cancer, diabetes, hepatic disorders, hemochromatosis, thalassemia, and alcoholism (12). After first indications of infection, death from septicemia typically occurs within one or two days (37, 68). Exposure of pre-existing or fresh wounds to seawater containing *V. vulnificus* can also lead to infection and a 25% mortality rate in people with wound infections has been observed (13). Wound infection is thought to occur as a consequence of seafood handling, fishing,

boating, swimming, or wading (2, 67). Virulence in *V. vulnificus* varies between strains. Strains isolated from hospital patients, referred to as 'clinical', are highly virulent whereas strains isolated from seawater, oysters, fish and sediments, referred to as 'environmental' are typically less virulent with some exceptions (23, 68, 189-193).

Due to the nature of infections (i.e. septicemia and wound infections) the biofilm form in the life cycle of *V. vulnificus* has largely been ignored. A few studies of biofilm formation by *V. vulnificus* have focused on the identification of proteins essential for biofilm development (pili, flagella, and outer membrane) (31, 46, 47, 134). One study, (230) examined the influence of nutrient levels, NaCl concentration, and temperature on biofilm formation and found that different broths and addition of glucose affected attachment of *V. vulnificus* as did incubation at different temperatures but not different salinities. In our previous study we demonstrated that *V. vulnificus* produces robust biofilms at 24°C indicating a potential role for biofilms in protection of this pathogen in the marine environment, and also produce biofilm at 37°C and at acidic pH, typical of the human host gastrointestinal tract.

As a follow up study to our previous work, we examined the expression of two important virulence factors, hemolysin/cytolysin (vvh) and capsular polysaccharide (group 1), during biofilm formation in *V. vulnificus*. We wanted to know if virulence of *V. vulnificus*, as represented by expression of genes encoding for these virulence factors, is affected by biofilm formation. Biofilm formation costs bacteria energy that could be used for cellular growth (135, 231, 232) and perhaps production of virulence factors. The up or down regulation of virulence genes during biofilm formation differ between

bacterial species. In some toxic *Escherichia coli* strains, several virulence-associated genes are up-regulated in biofilms versus planktonic forms including a pathogenicity island proposed to be associated with biofilm formation (233). However in *Staphylococcus aureus*, genes encoding for toxins and proteases are down-regulated in biofilm conditions (234).

Vvh facilitates iron acquisition by lyses of hemoglobin (109, 115). Purified vvh has also been found to increase vascular permeability, skin damage, and death rate in mice (35, 43). Moreover, the inoculation of vvh into mouse epithelial cells resulted in skin damage similar to those resulting from infection of *V. vulnificus* (35). Vvh also stimulates production of guanylate cyclase in the host, causing an increase in the amount of intracellular cyclic GMP, and consequently vasodilation (127, 128). Additionally, vvh functions as a “pore forming protein” in cellular membranes (129).

Capsular polysaccharide (CPS) is a key virulence factor for human and animal pathogens. CPS is considered as the most significant virulence determinant of *V. vulnificus* because it allows this pathogen to evade non-specific host immune response by avoiding complement mediated phagocytosis (37, 68, 235) and specific host immunity in which there is poor antibody response to the capsule (235). CPS, together with lipopolysaccharide, is thought to play an important role in septic shock formation by stimulating the expression of inflammation-associated cytokines (89). The presence of a capsule appears to be associated with more invasive *V. vulnificus*, since encapsulated forms are more commonly found among clinical isolates than among environmental isolates (28). CPS also plays a role in biofilm formation. CPS is important

for the initial coverage of surfaces by bacteria during adhesion and the construction of mature biofilm architecture (140, 236, 237) and in regulating size of the biofilm (238).

We examined the expression of *vvhA* and *CPS Operon allele 1* genes in a clinical strain of *V. vulnificus* during biofilm formation and in planktonic cells. *VvhA* encodes for *V. vulnificus*-specific hemolysin/cytolysin. There are two alleles that encode for Group I capsules in *V. vulnificus* and are strain-specific (86). Allele 1 is found in strains undergoing rapid phase switches between opaque (virulent) and translucent (avirulent) colonies. Allele 2 is found in strains that switch at lower rate and frequency. Allele specificity is also indicative of strain origin. Allele 1 is found in 87% of clinical strains (highly virulent) whereas allele 2 is found in 87% of environmental strains (less virulent).

Materials and methods

Brain Heart Infusion Broth (BHIB) mixed 1:1 with seawater (0.5 ml; 16 PSU; pH 7.5) in borosilicate glass test tubes was inoculated (approximately 10^3 cells) with a clinical strain of *Vibrio vulnificus* (FLA 8869) obtained from the U.S. FDA Gulf Coast Seafood Laboratory. Borosilicate tubes were used for biofilm formation and polyethylene tubes for planktonic growth of strain FLA. We found in previous experiments that polyethylene inhibits biofilm formation by *V. vulnificus*. Experimental design included 3 replicates for mRNA quantification of *vvhA* and *CPS allele 1* (86) genes during biofilm formation, 3 replicates for biofilm measurement, and 3 replicates for mRNA quantification of *vvhA* and *CPS allele 1* genes in planktonic FLA. Additional

replicates were used for bacterial cell density determination. Inoculated media was incubated at 24°C and 37°C on a shaker (120 rpm). Aliquots were sampled at 0, 4, 6, 8, 10, 12, and 24 hr.

RNA extraction. Total RNA was extracted from bacterial cells by first incubating with lysozyme (10 mg/ml) at 48°C for 40 mins, then followed by addition of 3% CTAB (cetyltrimethylammonium bromide) and incubated another 40 mins at 60°C with gentle mixing. The aqueous phase containing the nucleic acids was separated with chloroform:isoamyl and alcohol (24:1) and transferred to a new tube. Nucleic acids were precipitated with isopropanol (100%), collected by centrifugation and then treated with 80% ethanol to remove salts. After drying (3 mins) the nucleic acids were rehydrated in RNase/DNase free water (Sigma) and concentrations determined spectrophotometrically (NanoDrop ND-1000). To the greatest extent possible, precautions were taken to prevent RNase degradation of RNA. DNA removal with DNase I (Sigma) was followed by a reverse transcription reaction using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems. The resulting copy DNA (cDNA) was enumerated with quantitative PCR using a Smart Cycler (Cepheid) and virulence factor gene assays.

Quantitative PCR. *VvhA* was quantified using gene specific primers (vvhAF 5'-TGTTTATGGTGAGAACGGTGACA-3' and vvhAR 5'-TTCTTTATCTAGGCCCCAACTTG-3') and a probe labeled at the 5' end with Texas Red and at the 3' end with BHQ1 (5'-CCGTTAACCGAACCACCCGCAA-3') (78).

The reaction mixture (20 µl) consisted of 4 µl template DNA (different concentrations), 1 X PCR Buffer, 1 µM each primer, 250 nM probe, 200 nM dNTPs, 2.5 mM MgCl₂, 1 X BSA (bovine serum albumin), and 1 U Taq DNA polymerase (Roche). Thermal cycling was performed using a two-step PCR protocol: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min.

CPS allele 1 was quantified using gene specific primers (HP1F 5'TTTGGGAT TTGAAAGGCT TG-3' and HP1R 5'- TTTGGGATTTGAAAGGCTTG-3') adapted for qPCR from Han et al. (41) and Sybr Green intercalating dye. The reaction mixture (20 µl) consisted of 2-4 µl DNA template (different concentrations), 1 µM each primer, 2.5 mM MgCl₂, 1 X BSA and 10 µl of IQ Sybr Green Supermix (BioRad) that contains Taq DNA polymerase, and dNTPs. Thermal cycling was performed using a three-step PCR protocol: 30 cycles of 95°C for 90 s, annealing at 50°C for 60 s, and extension at 72°C for 60 s. Melt curve analysis was conducted from 60°C to 94°C at the increments of 0.2°C per second.

Standard curves were created in the Smart Cycler according to manufacturer instructions with the same primer sets (and probe) using cDNA from genomic DNA extracted from graduated concentrations (10¹, 10², 10³, 10⁴, 10⁵, 10⁶, 10⁷) of *V. vulnificus* strain FLA 8869. Counts (CFU) of *V. vulnificus* were generated by the standard curve and any dilutions were figured into the back calculation to the original DNA extraction volume.

Biofilm assay. The production of biofilm by different strains of *V. vulnificus* was determined using a modified crystal violet staining method (218). After the 48 hr incubation under the desired conditions, the culture medium in the borosilicate glass tubes was gently poured out as not to disturb the biofilm ring. Each tube was gently washed four times with 2% NaCl to remove any non-adhered cells. Crystal violet (0.1%; 850 µl) was added to tubes and incubated 40 minutes at room temperature. Afterwards, the unbound crystal violet was carefully decanted and tubes were rinsed thoroughly 3-4 times with 2% NaCl to remove the unbound dye. Biofilm-bound crystal violet was eluted with 600 µl of 98% ethanol for 10 minutes. Aliquots (100 µl) of the eluted dye were transferred to 96 well plates and absorbance was measured at 570 nm using a Synergy 2 Multi-Mode Microplate Reader (Biotek). When necessary, the eluted dye was diluted with ethanol for measurements and the final absorbance value was multiplied by the dilution factor after subtracting the OD reading from the control tubes.

Cell density. Bacterial density (cells/ml) was determined at OD_{600 nm} with a Synergy 2 Multi- Mode Microplate Reader (Biotek). To convert OD to cell counts, *V. vulnificus* in aliquots were stained with DAPI (4', 6-diamidino-2-phenylindole), filtered onto 0.2 µm polycarbonate membranes, and then counted with epifluorescence microscopy using a Zeiss Axioplan 2 microscope (Zeiss).

Results

Biofilm. By 4 h, biofilm rings were visible on glass tubes at the broth/air interface (Fig 20A). However, biofilm formation at 24°C and 37°C were markedly different. At 24°C, biofilm grew steadily from 4 h to 24 h reaching an optical density of extracted crystal violet (OD_{570 nm}) of 22 as cell replication entered the stationary phase. At 37°C biofilm formation peaked at 8 h, during the logarithmic growth phase of cell replication, with an OD_{570 nm} of extracted crystal violet of 16, and then decreased to an OD_{570 nm} of 8 by 24 h as cells entered the stationary phase of growth (Fig. 20A).

Cell replication. During biofilm formation at 24°C, cell density (Fig. 20A) increased rapidly from $3.1 \times 10^5 \pm 4.2 \times 10^4$ cells/ml at 6 h to $3.9 \times 10^6 \pm 1.40 \times 10^5$ cells/ml at 8 h (i.e. logarithmic growth phase), increased to $4.5 \times 10^6 \pm 7.0 \times 10^4$ cells/ml at 10 h, then decreased slightly to $4.1 \times 10^6 \pm 14.0 \times 10^4$ cells/ml at 24 hr (i.e. stationary growth phase). In planktonic form, cell density (Fig 20D) was a factor of 10 lower than the biofilm culture by 4 h ($3.0 \times 10^5 \pm 0$ cells/ml) but rapidly increased to $3.0 \times 10^6 \pm 2.8 \times 10^4$ cells/ml by 10 h (i.e. logarithmic phase) and remained at that density until 24 hr (stationary phase).

At 37°C, during biofilm formation, cell density (Fig. 21A) increased 10 fold from $1.0 \times 10^6 \pm 0$ cells/ml at 4 h to $1.5 \times 10^7 \pm 3.8 \times 10^5$ cells/ml at 24 h. Planktonic cell density was three-fold higher at 4 h than in the biofilm incubation (Fig. 21D). Cell density increased rapidly from $3.1 \times 10^6 \pm 8.3 \times 10^4$ cells/ml at 4 h to $2.4 \times 10^7 \pm 7.0 \times 10^3$ cells/ml at 12 h and then decreased to $2.1 \times 10^7 \pm 3.5 \times 10^5$ cells/ml by 24 h. Unlike biofilm production at 24°C, biofilm production at 37°C peaked at 8 h and then deteriorated steadily by 24 h.

Expression of *vvhA*. During biofilm formation at 24°C, *vvhA* gene expression (Fig. 20B) was low at 4 h ($1.2 \times 10^3 \pm 2.7 \times 10^2$ mRNA copies/ml) and 6 h ($1.4 \times 10^3 \pm 2.5 \times 10^2$ mRNA copies/ml) but increased three-fold by 8 h ($4.3 \times 10^3 \pm 2.9 \times 10^2$ mRNA copies/ml), increased another three-fold by 10 h ($1.2 \times 10^4 \pm 1.6 \times 10^3$ mRNA copies/ml) and increased another four-fold by 24 h ($5.1 \times 10^4 \pm 6.3 \times 10^3$ mRNA copies/ml). Expression of *vvhA* had a strong correlation with biofilm formation (linear regression $R^2=0.89$; $p<0.0001$). In the planktonic incubation, *vvhA* expression (Fig. 20E) increased from $1.0 \times 10^3 \pm 3.3 \times 10^1$ at 4 h to $8.3 \times 10^3 \pm 6.9 \times 10^2$ mRNA copies/ml at 10 hr. By 24 h, *vvhA* expression increased 17-fold to $1.4 \times 10^5 \pm 1.7 \times 10^4$ mRNA copies/ml. In the first 10 hours, rate of *vvhA* gene expression during biofilm formation was 1.9×10^3 mRNA copies/ml/h compared to 1.2×10^3 mRNA copies/ml/h in the planktonic incubation. However by 24 h *vvhA* expression in the planktonic incubation was more than double that of the biofilm incubation even though the cell density in the biofilm incubation was roughly 1.5 times higher. Highest expression of *vvhA*, in both the biofilm and planktonic incubations, occurred at 24 h. Normalization of mRNA copies/ml to individual bacterial cells (Table 25) revealed and up-regulation of *vvhA* expression at 4 h (1.07 ± 0.00 -fold), however between 4 and 24 h expression was down-regulated as much as (3.81 ± 0.39 -fold).

At 37°C, *vvhA* expression during biofilm formation (Fig. 21B) was already $3.0 \times 10^6 \pm 1.0 \times 10^5$ mRNA copies/ml at 4 h, 10^3 mRNA copies/ml higher than at 24°C, but then decreased gradually to $8.5 \times 10^5 \pm 2.9 \times 10^4$ mRNA copies/ml by 12 hr as the biofilm deteriorated. However, between 12 and 24 hr, as biofilm production continued to

decrease and cell density increased to $1.5 \times 10^7 \pm 3.8 \times 10^5$, expression of *vvhA* increased almost 4-fold to $3.1 \times 10^6 \pm 6.0 \times 10^3$ mRNA copies/ml. In the planktonic cells, *vvhA* expression was $2.4 \times 10^6 \pm 1.1 \times 10^5$ mRNA copies/ml at 4 h, similar to the biofilm incubation, and increased slightly to $3.0 \times 10^6 \pm 1.6 \times 10^5$ mRNA copies/ml by 12 h then decreased rapidly to $2.1 \times 10^5 \pm 9.2 \times 10^4$ mRNA copies/ml at 24 h as cell replication entered the stationary phase, likely due to decrease of nutrients in the incubation media (Fig. 21E). In contrast to the 24°C incubation, *vvhA* expression was up-regulated in the biofilm incubation (Table 25). The highest up regulation occurred at 4 h (3.78 ± 0.12 -fold) when biofilm production was lowest. At 12 h, as the biofilm began to deteriorate, there was a down-regulation (-1.15 ± 0.35 -fold) but by 24 h expression was up-regulated again (2.10 ± 0.00 -fold) as the biofilm continued to deteriorate and density of planktonic cells increased. There was no correlation between *vvhA* expression and biofilm formation at 37°C ($R^2=0.17$, $p=0.1401$).

Expression of *CPS allele 1*. During biofilm formation at 24°C, *CPS allele 1* expression (Fig. 1C) decreased during logarithmic phase growth of cells (between 4 and 12 h; $1.0 \times 10^5 \pm 4.6 \times 10^3$ to $4.1 \times 10^3 \pm 1.6 \times 10^3$ mRNA copies/ml, respectively) then increased approximately 400-fold to $1.5 \times 10^6 \pm 1.0 \times 10^5$ mRNA copies/ml by 24 h. However, in the planktonic culture, *CPS allele 1* expression (Fig. 1F) increased during logarithmic phase (between 4 and 8 h; $1.2 \times 10^6 \pm 1.5 \times 10^5$ mRNA copies/ml to $1.7 \times 10^6 \pm 3.0 \times 10^5$ mRNA copies/ml, respectively) but then decreased eight-fold to $5.9 \times 10^5 \pm 7.1 \times 10^4$ mRNA units/ml by 24 h. In the biofilm incubation, normalization of

mRNA copies/ml per cell revealed an up-regulation of 1.5 ± 0.18 -fold at 4 h (Table 25). At 8 h there was a major down-regulation of 74.42 ± 42.16 -fold and a further down-regulation of 453.76 ± 193.32 -fold by 12 h. However, by 24 h, expression was up-regulated 1.88 ± 1.07 -fold as biofilm production peaked. Expression of *CPS allele 1* was not correlated with biofilm production ($R^2=0.0363$, $p=0.5982$).

At 37°C, *CPS allele 1* expression during biofilm formation (Fig. 21C) was high and constant at $\sim 4.50 \times 10^7$ mRNA copies/ml from 4 to 8 h but then decreased gradually to $3.94 \times 10^7 \pm 4.24 \times 10^5$ by 24 h as the biofilm deteriorated. In the planktonic incubation, expression of *CPS allele I* was similar to that in the biofilm incubation at 4 h ($4.48 \times 10^7 \pm 8.75 \times 10^5$ mRNA copies/ml) but decreased rapidly between 4 and 8 h to $3.31 \times 10^5 \pm 2.74 \times 10^4$ mRNA copies/ml and decreased further to $1.40 \times 10^2 \pm 3.07 \times 10^1$ mRNA copies/ml by 24 hr as the cells entered stationary phase. In the biofilm incubation *CPS allele 1* expression was up-regulated 1.69 ± 0.05 -fold at 4 h and increased to an up-regulation of $4.03 \times 10^5 \pm 4.34 \times 10^3$ by 24 h compared to the planktonic incubation.

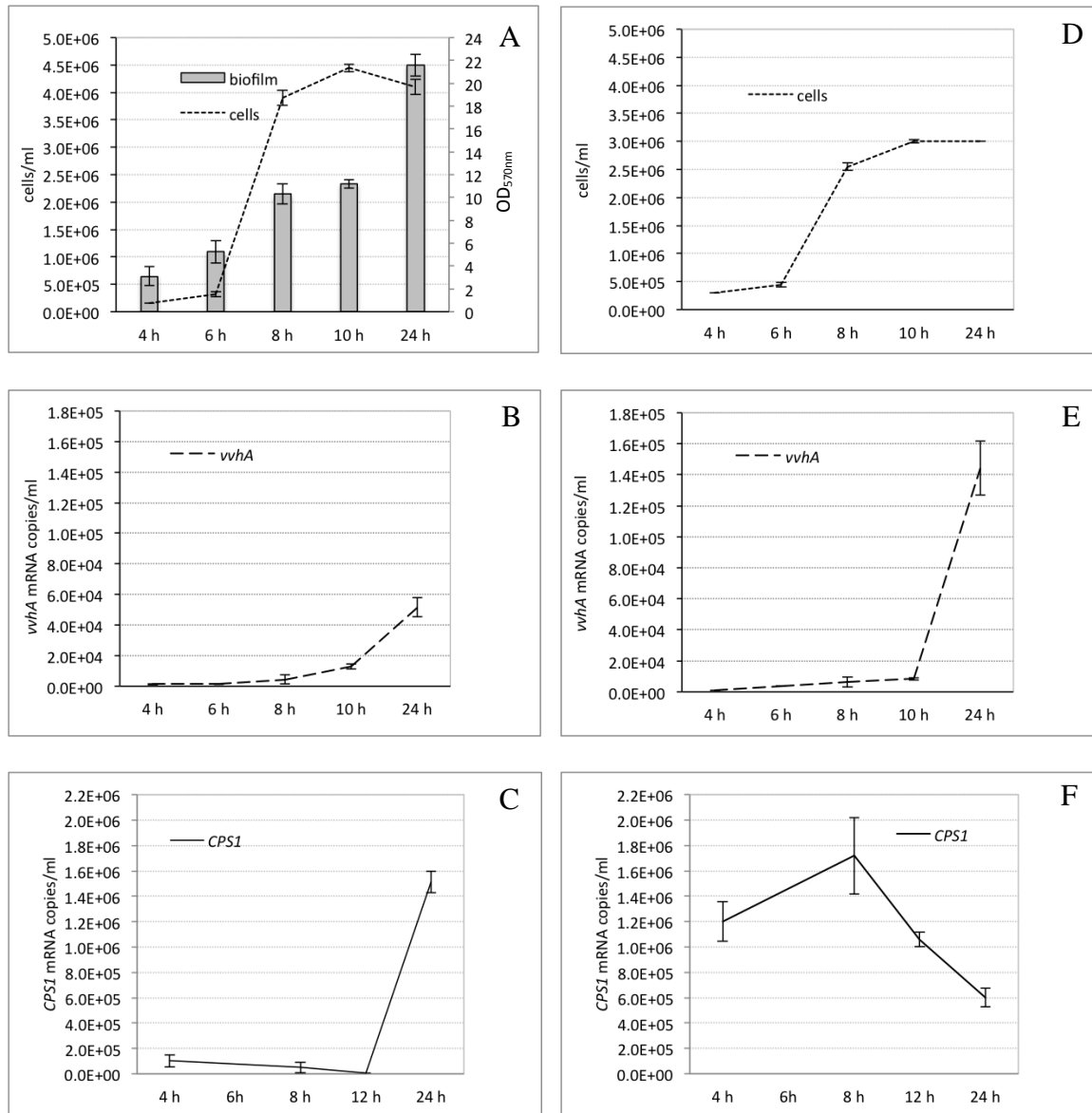


Figure 20. *V. vulnificus* isolate FLA 8869 incubated at 24°C for 24 h: A) cell density (cells/ml) and biofilm formation, B) *vvhA* expression (mRNA copies/ml) and C) *CPS* allele 1 expression (mRNA copies/ml) during biofilm formation; D) cell density (cells/ml), E) *vvhA* expression (mRNA copies/ml) and F) *CPS* allele 1 expression (mRNA copies/ml) in planktonic form.

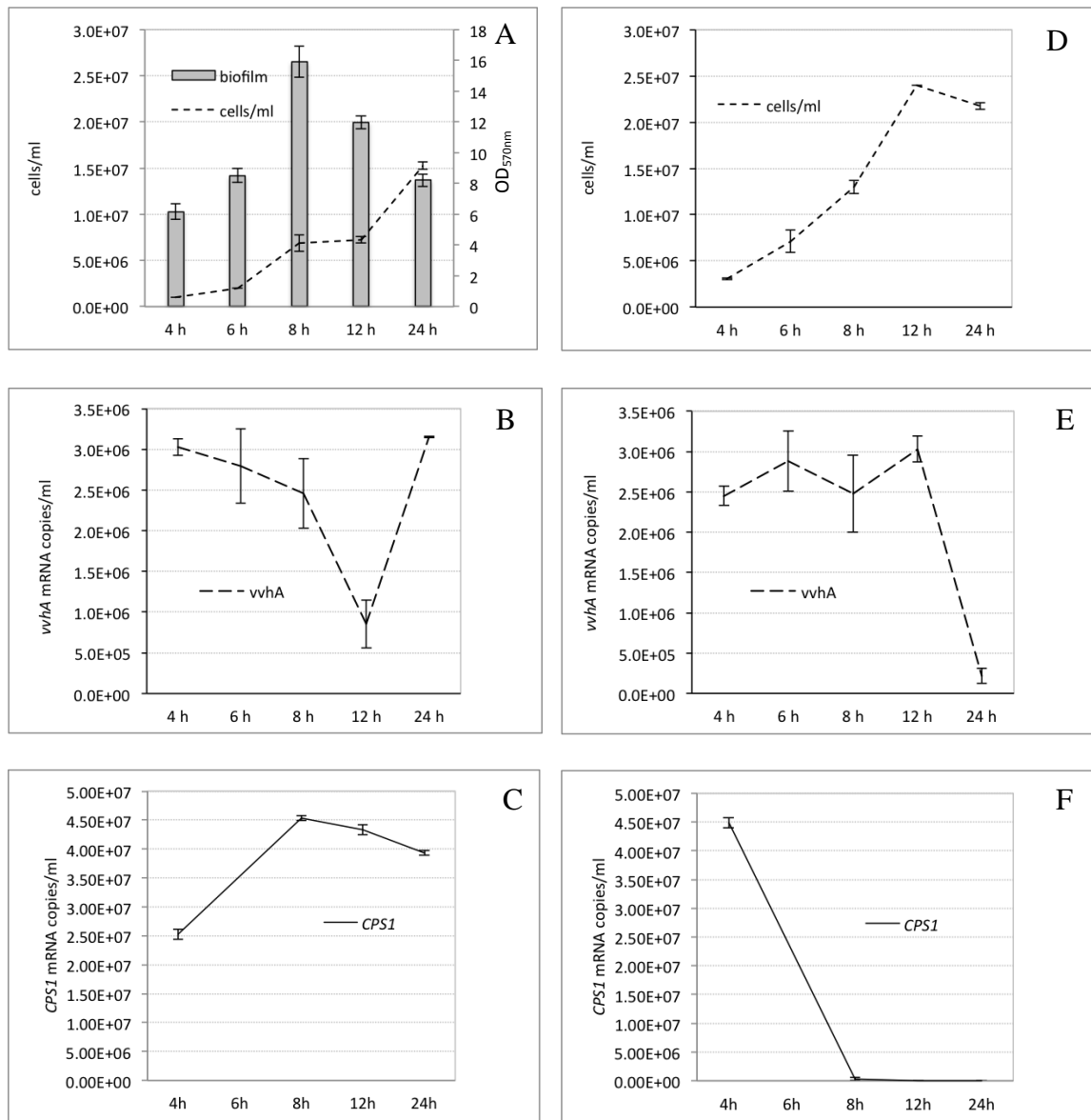


Figure 21. *V. vulnificus* isolate FLA 8869 incubated at 37°C for 24 h: A) cell density (cells/ml) and biofilm formation, B) *vvhA* expression (mRNA copies/ml) and C) *CPS allele 1* expression (mRNA copies/ml) during biofilm formation; D) cell density (cells/ml), E) *vvhA* expression (mRNA copies/ml) and F) *CPS allele 1* expression (mRNA copies/ml) in planktonic form.

Table 25. Up (+) or down (-) regulation (fold difference) of *vvhA* and *CPS allele 1* expression normalized to individual cells during biofilm formation.

Temperature	Hour	<i>vvhA</i>	<i>CPS allele 1</i>
24°C	4	+1.07±0.00	+1.50±0.18
	6	-3.43±1.75	ND
	8	-3.50±2.96	-74.42±42.16
	10	-0.97±0.13	-453.76±193.32
	24	-3.81±0.39	+1.88±1.07
37°C	4	+3.78±0.12	+1.69±0.05
	6	+3.45±0.56	ND
	8	+1.87±0.63	+258.83±2.42
	12	-1.15±0.35	+1.25×10 ⁴ ±1.23×10 ²
	24	+2.10±0.00	+4.03×10 ⁵ ±4.34×10 ³

Discussion

Here we report a comparative transcript analysis of the *vvhA* and *CPS allele 1* expression of clinical *V. vulnificus* cells grown in a biofilm and in cells grown planktonically at 24°C and 37°C. We compared the expression of these genes at two temperatures because, in our previous study, *V. vulnificus* was shown to produce robust biofilms at 24°C typical of the marine environment and produced moderate biofilms at 37°C, the temperature of the human host.

During biofilm formation at 24°C, *vvhA* expression per cell was down-regulated by almost 4-fold at 24 hr compared to expression in planktonic cells likely because cellular energy was devoted to biofilm formation rather than toxin production.

Interestingly, *vvhA* expression was positively correlated with biofilm formation ($R^2=89$; $p<0.0001$) indicating a possible co-regulation. It is important to note that even though expression of *vvhA* was down-regulated during biofilm formation at 24°C, it was nonetheless expressed and demonstrates that the biofilm form of *V. vulnificus* is still virulent, albeit less virulent than planktonic cells.

At 37°C, *vvhA* expression in the biofilm incubation at 4 h was roughly 3-fold higher than expression at 24°C. And, in contrast to the down-regulation observed in the 24°C incubation, expression of *vvhA* per cell during biofilm formation at 37°C was up-regulated compared to planktonic cells (Table 25). Like *V. cholerae*, *V. vulnificus* appears to be more virulent in biofilms than in planktonic form at the temperature of the human host (50) and could explain the rapid infection rate of 24 to 48 h. Interestingly, *vvhA* expression per cell increased 3-fold between 12 and 24 h as the biofilm deteriorated and planktonic cell density increased. Again, the decrease in *vvhA* expression as the biofilm deteriorated indicates a possible co-regulation of biofilm and hemolysin/cytolysin production. In *V. cholerae*, cholera toxin and biofilm production are positively co-regulated by the VieS/A/B signal transduction system (239, 240). And in *Bordetella bronchiseptica*, another human pathogen, a two component signal transduction system (BvgA/S) controls both biofilm formation and virulence (241). The up-regulation of *vvhA* expression at 37°C compared to the down-regulation at 24°C during biofilm formation indicates that production of hemolysin/cytolysin is stimulated at higher temperature (i.e. that of the human host) and emphasizes that the biofilm form may be the preferred form of *V. vulnificus* in the human host.

CPS has been demonstrated to be essential for adherence of bacterial cells to surfaces (236, 237). We observed that in the biofilm incubation at 24°C, expression of *CPS allele 1* was up-regulated at 4 h as the cells were attaching to the glass. Interestingly, expression at 24°C was down-regulated as the biofilm developed further indicating that CPS is not essential for intermediate biofilm formation. But by 24 h as the biofilm matured, *CPS allele 1* expression was up-regulated again which seems to confirm the suggestion by Lee et al. (238) that CPS plays a critical role in determining *V. vulnificus* biofilm size by restricting continual growth of mature biofilms. At 24°C, there was no correlation between biofilm formation and *CPS allele 1* expression however in early and late stages of biofilm formation CPS appears to be an important component of biofilm architecture in *V. vulnificus*, as indicated by our data. In the planktonic incubation, *CPS allele 1* expression was highest in the first 8 h during logarithmic phase, and then decreased 8-fold by 24 h as cells entered the stationary phase. This pattern is similar to a study by Wright et al. (97) who observed that expression of CPS varied with the growth phase, increasing during logarithmic growth and declining in stationary culture. Differences between expression of CPS allele 1 in biofilm and planktonic incubations provide insights into the different functions of CPS.

Compared to the 24°C incubation, expression of the *CPS allele 1* gene at 37°C was ~50× higher during biofilm formation indicating that CPS is an important component of biofilm in *V. vulnificus*. The up-regulation of *CPS allele 1* expression at the peak of biofilm formation and during deterioration of the biofilm agrees with our observation at 24°C that CPS controls the size of the mature biofilm (Fig. 21C and Table

25). Interestingly, *CPS allele 1* expression in the planktonic cells decreased as cell number increased. This is counter to what is known about CPS as protection from host immune response. One would expect CPS production to increase with increasing cell replication. This observation and the up-regulation of *CPS allele 1* in the biofilm incubation emphasize the potential preference of the biofilm form of *V. vulnificus* in the human host rather than planktonic form. Preference for the biofilm form could also explain the increased virulence of clinical strains versus environmental strains in mice (23).

The results of this study differ from a previous study of biofilm formation in *V. vulnificus*. Joseph and Wright (45) observed that CPS inhibited adherence of planktonic cells to glass as well as biofilm formation. These differences may be due to alterations in incubation conditions (i.e. temperature, static vs. shaking, substrate). We observed the formation of thick, robust biofilms during shaking as compared to static incubation that was used by Joseph and Wright (45). And, strain differences also affect biofilm formation. In our previous study, significant strain to strain differences in biofilm production by clinical strains of *V. vulnificus*.

Conclusions. The genes encoding for the virulence factors, hemolysin/cytolysin (i.e. *vvhA*) and capsular polysaccharide (i.e. *CPS allele 1*), during biofilm formation revealed that their expression was influenced by maturity of the biofilm as well as by temperature. Expression was also up-regulated at 37°C during biofilm formation indicating that biofilm may be the preferred form of *V. vulnificus* in the human host. In

addition to avoidance of host immune defenses, CPS is also an important component of *V. vulnificus* biofilm and affects adherence and size of biofilm. The results of this study indicate that the biofilm form may be important in the life phases of *V. vulnificus* both in the marine environment and in the human host.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Understanding the environmental factors influencing virulence of *V. vulnificus* is of utmost importance in reducing human exposure to this deadly pathogen. Knowledge of the seasonal occurrence and life cycle offers clues to predicting the presence of more virulent clinical and less virulent environmental strains in oysters, other sea life, and seawater.

Seasonal variation of virulence

Chapter III examined how seasonal changes in temperature and salinity affect virulence of *V. vulnificus* in oysters. Since the discovery of more virulent clinical and less virulent environmental strains of *V. vulnificus*, identification of virulence factors to differentiate these strains has been the focus of numerous studies (37, 86, 97, 100, 178-180). Molecular methods such as PCR assays targeting virulence indicator genes proved to be the most effective for differentiation (24, 25, 40, 181-183). However these assays were used primarily with isolated strains and enumeration depended upon enrichment and streaking onto selective-differential agars. The development of quantitative PCR assays for virulence indicator genes has allowed for not only detection but also enumeration of clinical and environmental strains in environmental samples, thus eliminating the need for isolation. In Chapter III, quantitative PCR assays for 16S rRNA type A/B and the clinical allele of the virulence correlating gene (*vcgC*) were used to

determine the seasonal occurrence of clinical and environmental strains of *V. vulnificus* in oysters rather than screening individual isolate cultures for virulence genes. 16S rRNA type A targets environmental strains and 16S rRNA type B targets clinical strains. *VcgC* targets clinical strains. The null hypothesis that there is no seasonal variation of clinical and environmental strains in environmental samples was disproven. Strong seasonality of clinical strains of *V. vulnificus* environmental strains was observed in oysters. Environmental strains dominated the pool of *V. vulnificus* from April to June as water temperatures gradually rose from 18 to 30°C and salinities increased from 22 to 27 PSU but were then succeeded by clinical strains from July through September when water temperatures were sustained at 30°C or above and salinities rose above 27 PSU. The hypothesis that the *vcgC* gene can accurately detect clinical strains in environmental samples was proven. The occurrence of the *vcgC* gene correlated strongly with occurrence of the 16S rRNA type B allele, the other virulence indicator for clinical strains. The use of two virulence indicators offered more conclusive evidence for detection of clinical strains. The seasonality and prevalence of clinical strains during warmer months observed in this study is in good agreement with Lin and Schwarz (53) who examined 16S rRNA allele types in Galveston Bay oysters and seawater. In contrast, Warner and Oliver (182), who examined the seasonal occurrence of clinical and environmental isolates in oysters and seawater from North Carolina and Florida, found equal numbers of both strains in seawater but a predominance of environmental strains in oysters throughout the year. Like the current study, they did find that clinical strains increased in number in both oysters and seawater as water temperatures increased.

However, another study (242) examined market oysters from around the U.S. and found higher percentages of clinical strains in Gulf Coast oysters (up to 60%), where seawater temperatures are warmer, than in East Coast oysters (up to 18%) throughout the year. This study concluded that most foodborne *V. vulnificus* infections in the U.S. were from Gulf Coast oysters. This is a reasonable conclusion since there are also higher numbers of *V. vulnificus* in Gulf Coast oysters ($>10^3$ per g) than in East Coast oysters (10^2 per g) (15) due to warmer water temperatures.

Conclusions. The current study and others demonstrate conclusively that temperature and salinity have a strong influence on selection for clinical or environmental strains of *V. vulnificus*. This finding has implications for increase of more virulent strains of vibrios due to global climate change (243-246), which have already been observed in *V. cholerae* (247).

Selection of enrichment media for more virulent strains

Most studies of the virulence of *V. vulnificus* in environmental samples have relied upon isolate cultures. Isolation methods typically involve an overnight enrichment in alkaline peptone water (APW), having a salinity of 10 PSU, that selects for halophilic bacteria (197). Enrichment is followed by streaking onto selective-differential agars and picking of single isolate colonies. In Chapter III, oyster samples were enriched for 20 h in APW prior to screening with quantitative PCR for virulence genes. This enrichment brought into question the potential for APW to select for clinical or environmental

strains of *V. vulnificus*, thus skewing the data. The potential selection for one strain or the other by different enrichment media was tested in Chapter IV. APW, Brain Heart Infusion Broth (BHIB), and Luria Bertani Broth (LB) were adjusted to the same salinity and inoculated with Galveston Bay seawater samples collected in the spring (March), late summer (September), and fall (November). This study found that BHIB with its higher iron content selected for clinical strains however the starting inoculum of clinical strains was important. In March when the occurrence of clinical strains was low or non-detectable, there was no significant enrichment. In September and November when the occurrence of clinical strains in seawater was presumably higher, significant preferential selection by BHIB was observed. Laboratory testing with isolate cultures found that enrichment with APW did not alter the ratio of environmental to clinical strains but BHIB preferentially selected for the clinical isolate. Therefore, percentages of clinical out of total *V. vulnificus* observed by enrichment with APW were a truer representation of the natural population in the seawater samples. This finding disproved the hypothesis that APW does not select equally for clinical and environmental strains. And the hypothesis that BHIB with its high iron content selects preferentially for clinical strains was proven.

Conclusions. The selective enrichment of BHIB for clinical strains indicates that iron plays an important role in the infection of the human host. Seasonality of *Vibrio vulnificus* clinical and environmental isolates influenced the starting inoculum and ultimately the selective enrichment for clinical strains in all media tested.

Biofilm formation

Chapter V examined biofilm production of clinical versus environmental isolate strains of *V. vulnificus* under different temperature, pH, and iron concentration conditions. Clinical strains were found to produce significantly more biofilm than environmental strains at 24°C versus 30°C or 37°C, thus disproving the hypothesis that clinical strains would produce more biofilm at 37°C, the temperature of the human host. The formation of robust biofilms at 24°C indicates that the biofilm form may be important for protection of clinical strains in the marine environment. *V. cholerae* forms thick biofilms 15°C and 25°C, temperatures encountered in the aquatic environment (159). *V. cholerae* also produces biofilm *in vivo* within the human host (49) and *in vitro* at 37°C but they are thinner and less structured (160). And, biofilm formation by *V. cholerae* is stimulated bile acids (162). Similarly, there was a significant trend for clinical strains of *V. vulnificus* to form more biofilm at acidic pH (5.5) than environmental strains, at both 24°C and 37°C, indicating that biofilm may be important for survival of clinical strains in the gastrointestinal tract of the human host. This finding supports the hypothesis that *V. vulnificus* produces more biofilm at acidic pH than at neutral or alkaline pH.

Conclusions. Higher biofilm production appears to be a trait of clinical strains and could be considered a virulence factor. This study revealed that the biofilm form may be important in the life cycle and ecology of *V. vulnificus*, in particular for clinical strains.

Expression of virulence factors in biofilms

Chapter VI examined the expression of hemolysin/cytolysin (i.e. *vvhA*) and CPS (i.e. *CPS allele 1*) genes during biofilm formation. Expression was influenced by maturity of the biofilm as well as by temperature. At 24°C, *vvhA* expression was down-regulated but at 37°C it was up-regulated by 10⁵-fold indicating that it is stimulated by higher temperature (i.e. that of the human host). The up-regulation at 37°C supports the hypothesis that *V. vulnificus* expression of the hemolysin/cytolysin gene (*vvhA*) will be higher during biofilm formation than in planktonic cells.

CPS allele 1 expression at 24°C was up-regulated during the attachment phase of the biofilm, down-regulated during the growth phases of the biofilm and then up-regulated again as the biofilm matured to control size of the biofilm. A similar pattern was observed at 37°C. Compared to planktonic cells, expression of CPS allele 1 was up-regulated during biofilm formation and even deterioration emphasizing the importance of CPS in these processes. These findings disprove the hypothesis that *CPS allele 1* will be suppressed during biofilm formation and increased in planktonic forms of *V. vulnificus*.

Conclusions. The up regulation of expression of both genes at 37°C during biofilm formation indicates that biofilm may be the preferred form of *V. vulnificus* in the human host. In addition to avoidance of host immune defenses, CPS is also an important component of *V. vulnificus* biofilm and affects adherence and size of biofilm.

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